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(57) Abstract

The present invention involves the creation of defined chromosomal deficiencies, inversions and duplications using Cre recombinase in ES cells transmitted into the mouse germ line. These chromosomal reconstructions can extend up to 3-4 cM. Chromosomal rearrangements are the major cause of inherited human disease and fetal loss. Additionally, translocations and deletions are recognized as major genetic changes that are causally involved in neoplasia. Chromosomal variants such as deletions and inversions are exploited commonly as genetic tools in organisms such as Drosophila. Mice with defined regions of segmental haploidy are useful for genetic screening and allow accurate models of human chromosomal diseases to be generated.

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CHROMOSOMAL REARRANGEMENT BY INSERTION OF TWO RECOMBINATION SUBSTRATES

The present invention was made utilizing funds of the United States Government. The U.S. Government is entitled to certain rights under this invention.

Background of the Invention

Technical Field of the Invention

The present invention involves the creation of defined chromosomal deficiencies, inversions and duplications using Cre recombinase in embryonic stem cells and transmitted into the mouse germ line. In the present invention, these chromosomal reconstructions can extend up to 3-4 cM. Chromosomal rearrangements are the major cause of inherited human disease and fetal loss. Further, chromosomal translocations and deletions are recognized as major genetic changes that are causally involved in neoplasia. Chromosomal variants such as deletions and inversions are exploited commonly as genetic tools in diploid organisms such as *Drosophila*. In diploid organisms, such deficiencies are exploited in genetic screens because a small portion of the genome is functionally hemizygous. Thus, a mutation which would normally be recessive and masked by the wildtype allele in a diploid context will be dominant and detectable in the haploid state. In the mouse, deficiencies have not, up to now, been available generally; thus, screens for recessive mutations are nonexistent or particularly cumbersome. However, the present invention provides methods to engineer mice and cell lines with defined regions of segmental haploidy. Such mice are useful for genetic screening and provide accurate models of human chromosomal diseases.

The Prior Art

Inherited chromosomal rearrangements such as inversions, duplications and deficiencies are responsible for a significant fraction of human congenital disease. Chromosomal changes also occur somatically and are associated with neoplastic disease. Defining the causal genetic alteration in a region of the genome associated with chromosomal rearrangements can be relatively straightforward if the affected gene lies in the breakpoint of an inversion or translocation. However, in cases of duplications and deficiencies, the specific

genetic lesion(s) associated with pathological chromosomal changes are much harder to identify. Still, the generation of animal models that accurately recapitulate the genetic lesion would facilitate the study of disease and could be very helpful in the efforts to dissect specific gene-function relationships in multigene syndromes.

In diploid organisms such as *Drosophila*, chromosomal deficiencies are commonly exploited in genetic screens because a small portion of the genome is functionally hemizygous. Thus, a mutation which would be recessive and masked by the wildtype allele in the diploid context will be dominant and therefore readily detectable in the haploid state. In the mouse, deficiencies are not available generally. Despite the limited number of deficiencies available in the mouse, the potential for the detailed analysis of a genetic interval using these deficiencies has been demonstrated clearly. *See* Holdener-Kenny, et al., *BioEssays*, 14:831-39 (1992), which is hereby incorporated by reference.

Deficiencies that are available currently in the mouse genome were generated at random using ionizing irradiation. Although conventional gene targeting technology in embryonic stem (ES) cells can generate virtually any type of mutation, including deletions of up to 20 kb, it has not been possible to delete substantially larger fragments by using standard methodology. Likewise, the technology required to construct large inversions and duplications has not been established.

One mechanism by which chromosomes may be engineered is by the use of Cre recombinase. Cre recombinase has been used in mammalian cell lines and *in vivo* to delete or invert sequences between the 34 base pair recognition sequences, *loxP* sites, placed a few kb apart on the same chromosome. The recombination is initiated by Cre proteins which bind to 13-bp inverted regions in the *loxP* sites and promote synapses or joining of a pair of sites. Next, the Cre proteins catalyze strand exchange between the pair of sites within an asymmetric 8-bp central spacer sequence by concerted cleavage and rejoining reactions, involving a transient DNA-protein covalent linkage. Smith, et al., *Nature Genetics*, 9:376-385 (1995); Gu, et al., *Science*, 265:103-06 (1994) and Sauer, *Nucl. Acids Res.*, 17:147-61 (1989) (both of these references are hereby incorporated by reference). Additionally, recombinases have been used to induce

mitotic recombination between homologous and non-homologous chromosomes in *Drosophila*, plants and mammalian cells. Embryonic stem cell technology has become a powerful tool for defining the function of mammalian genes, but mainly has been restricted to the mutation of single genes. Replacement vectors have been used to construct deletions of up to 19 kb; however, utilizing the same strategy to construct larger deletions (>60 kb) has not been successful. In the present invention, the generation and direct selection of deletions, duplications and inversions, ranging from 90 kb to 3-4cM, in ES cells is demonstrated.

Summary of the Invention

The method of the present invention is based on consecutive gene targeting of two recombination substrates to the deletion endpoints and the subsequent induction of recombination mediated by the Cre recombinase. This method generates a positive selectable marker allowing for the direct selection of clones with the desired chromosome structures. Despite the multitude of steps involved in generating these rearrangements in ES cells, deletion and duplication alleles have been transmitted into the mouse genome.

One object of the present invention is a method for causing a large-scale chromosomal rearrangement by first deleting a portion of genetic material.

An additional object of the present invention is a targeting vector system capable of inserting into two endpoint regions constraining a desired chromosomal deletion.

Thus in accomplishing the foregoing objects, there is provided in accordance with one aspect of the present invention a method for deleting a selected region of genetic material in cells comprising the steps of: inserting a first selection cassette at a 5' end of said selected region using conventional gene targeting methods, said first selection cassette comprising a first selectable marker, a first *loxP* recombination site, and a first portion of a second selectable marker; selecting cells expressing said first selectable marker; inserting a second selection cassette at a 3' end of said selected region using conventional gene targeting methods, said second selection cassette comprising a third selectable marker, a second *loxP* recombination site, and a remaining portion of said second selectable marker; selecting cells expressing said third selectable marker;

expressing Cre recombinase to produce recombination between said first and second *loxP* sites; and selecting cells expressing said second selectable marker.

Specific embodiments of the above method can include a puromycin resistance gene as the first selectable marker, a functional *Hprt* gene as the second selectable marker, and a neomycin resistance gene as the third selectable marker. Numerous other selectable markers will work, their presence in the particular deletion strategy is merely to aid cell selection. In other preferred embodiments, the first selectable marker is a puromycin resistance gene. In still other preferred embodiments, the second selectable marker is a functional *Hprt* gene. And in still other preferred embodiments, the third selectable marker is a neomycin resistance gene.

In still other preferred embodiments, the cells referred to above are embryonic stem cells, though significant, they need not be stem cells. In other preferred embodiments, the cells are embryonic stem cells, and said cells develop into mice. And in yet other preferred embodiments, the cells are embryonic stem cells, and said cells are maintained as cell lines.

In yet another preferred embodiment, a viral vector is used to replace either or both native sequences of DNA. In one embodiment, this virus is a retrovirus. In yet another embodiment, the viral vector referred to above has a provirus structure comprising a cassette in turn comprising: an *hprtΔ5'* cassette, a *loxP* site, and a puromycin resistance gene.

In yet another particularly preferred embodiment, the method for deleting a portion of chromosomal material in cells wherein the targeting vectors are a first targeting vector for replacing said first native sequence of DNA at said 5' end, comprising: a genomic insert cloned into the vector of about 7.5 kb; a tyrosinase minigene; a Neo^r gene; a 5' *hprt* fragment; and a *loxP* site embedded into said *hprt* fragment; and a second targeting vector for replacing said second native sequence of DNA at said 3' end, comprising: a genomic insert cloned into the vector of about 8.5 kb; a K14-Agouti gene; a Puro^r gene; a 3' *hprt* fragment; and a *loxP* site embedded into said *hprt* fragment.

In one particularly preferred embodiment of the second aspect of the present invention, there is provided a replacement vector system comprising a first targeting vector for replacing said first native sequence of DNA at said 5'

end, comprising a genomic insert cloned into the vector of about 7.5 kb; a tyrosinase minigene; a Neo^r gene; a 5' *hprt* fragment; and a *loxP* site embedded into said *hprt* fragment; and a second targeting vector for replacing said second native sequence of DNA at said 3' end, comprising: a genomic insert cloned into the vector of about 8.5 kb; a K14-Agouti gene; a Puro^r gene; a 3' *hprt* fragment; and a *loxP* site embedded into said *hprt* fragment.

Brief Description of the Figures

Figure 1: 1A: depicts the *Hprt-loxP* minigene cassette; 1B: depicts the *hprt*Δ*5'* and *hprt*Δ*3'* recombination substrates; 1C: outlines the general strategy for Cre-induced, targeted genomic rearrangements illustrated at the *HoxB* cluster--only the intrachromosomal pathway is shown; 1D: demonstrates alternative orientations (A or B) of the recombination substrates at the *E2DH* and *Gastrin* loci; 1E: shows chromosomal alterations induced by Cre recombinase for the different orientations of the minigenes in *cis* and *trans*.

Figure 2: Shows the results of a Southern blot analysis of the chromosomal engineering technology used to delete the *HoxB* cluster. A-C demonstrates the interpretation of, and D-F shows the actual Southern blot data from wildtype (wt), double targeted (dt) or HAT resistant ES cell clones (lanes 1 and 2). M1 and M2 are *HindIII*-cut and *BstEII*-cut lambda DNA molecular weight markers, respectively. *Hoxb*-1 is located in a 7.2 kb *NheI* fragment detected with probe *a* (2A and 2D; this allele is present in all of the lanes). Targeting of the *hprt*Δ*3'*-neo cassette to the *Hoxb-1* locus generates a novel 10.2kb *NheI* restriction fragment detected with probe *a* (2B and 2D dt). *Hoxb*-9 is located on a 16 kb *NheI* restriction fragment detected with probe *b* (2A and 2E; this allele is present in all of the lanes). Targeting of the *hprt*Δ*5'*-puro to the *Hoxb*-9 gene generates a novel 20.4 kb *NheI* restriction fragment detected with probe *b* (2B and 2E dt). Cre-induced recombination brings together the *hprt*Δ*5'* and *hprt*Δ*3'* and produces a *NheI* 18.2kb deletion-specific junction fragment detected by both probes *a* and *b* (2C, 2D 1 and 2). Probe *c*, located in the deletion region, shows a dosage difference in Panel 2F, 1 and 2, compared to the wt and dt lanes. Probe *a* is a 0.7 kb *RsaI* fragment located approximately 3kb downstream of *Hoxb*-1

exon 2; probe *b* is a 1 kb *RsaI* fragment located approximately 5 kb upstream of *Hoxb*-1 exon 1. P and N represent the puromycin and neomycin selection cassettes.

Figure 3: 3A: depicts mouse chromosome 11, and the loci used as endpoints for the chromosome engineering are illustrated. 3B: shows *NheI* restriction sites (N) and fragment lengths around the *E2DH* and *Gastrin* loci. 3C: shows chromosome 11 with both the *E2DH* and *Gastrin* loci targeted with the *hprt*Δ*5′* and *hprt*Δ*3′* vectors, respectively. For clarity, only the *cis* configuration and the A orientation are shown. 3D: shows the structure of the deletion, duplication and inversion alleles. The sizes of the diagnostic restriction fragments and probes used to detect these alleles are indicated. The deletion, duplication and inversion alleles are derived from the double targeted chromosome in the AA, BB and AB configurations, respectively. 3E: shows the results of Southern blots which confirm the structure of the recombinant chromosomes. The probes used with each blot *(a, b, c* or *d)* are indicated beneath each panel and on the diagrams of the various alleles. The lanes are coded as follows: wildtype (wt), double targeted (dt), deletion (del), duplication (dup) and inversion (inv).

Figure 4: G_2 *trans* recombination between homologous chromosomes. 4A: depicts individual sister chromatids from chromosome homologues still joined at the centromere. One chromosome is illustrated with *hprt*Δ*5′*, the neomycin resistance gene (N) used for targeting. The other homologue was targeted with *hprt*Δ*3′* cassette linked to the puromycin resistance gene (P). Cre-induced recombination between *loxP* sites on sister chromatids from different homologues is illustrated by an X. B: illustrates the recombinant structure of the sister chromatids. The individual chromatids are numbered 1-4. Chromatid 3 carries the reconstructed *Hprt* minigene and the deletion. 4C: shows the results of HAT selection for chromatid 3 which will segregate with either chromatid 1 or 2 which carries only the *neo* or both the *neo* and *puro* cassettes, respectively. The chromatid 2+3 segregant carries a duplication and deletion (genetically balanced) and is indistinguishable from the G_1 inter-chromosomal product. The chromatid

1+3 product carries the deletion and the original single targeted chromosome; this can only have arisen via the G_2 pathway.

Figure 5: Gene dosage analysis and segregation of the deletion and duplication chromosomes through the mouse germ line are demonstrated: Lane 1: wildtype allele (AB2.2 ES cell line); Lane 2: ES cell clone with the duplication and deletion (genetically balanced); Lanes 3 to 6: transmission and segregation of the duplication and deletion alleles in the progeny of a chimeric male constructed from the cells shown in lane 2. Lanes 3 and 4 show mice with the deletion, lanes 5 and 6 show mice with the duplication; the increase or decrease in intensity of the 9.0 kb fragment relative to the 2.0 kb control fragment is consistent with junction fragment analysis of the inheritance of the duplication or deletion alleles from these mice (data not shown). Lanes 7 and 8 show mice from heterozygous mating homozygous for the duplication allele which is evident from the increased intensity of the 9.0 kb fragment.

Figure 6: Deletion of two 3-4cM intervals on mouse chromosome 11 is shown. 6A depicts mouse chromosome 11. The shaded bars indicate the intervals which are to be deleted. *Hox B, E2DH* and *Wnt3* are the loci which serve as the deletion endpoints. SBC (Sporadic Breast Cancer) loci are indicated by the black bars. These loci are the putative location of tumor suppressor genes based on the analysis of loss of heterozygosity in breast cancer. 6B depicts a double-targeted chromosome which has been targeted with the *hprtΔ3'* cassettes to the *Hoxb9* and *E2DH* genes or to the *E2DH* and *Wnt3* genes. The A orientation *E2DH*-targeted clones were used with the *HoxB-E2DH* deletion and the B orientation clones were used with the *E2DH-Wnt3* deletion. Only the orientations of the *hprtΔ5'* cassette which give the deletion products are illustrated. The vertical bars represent *NheI* sites; the sizes of the fragments are indicated and the probes are indicated by shaded boxes labelled *c* and *d*. 6C shows the structure of deletion alleles. Diagnostic *NheI* fragments for the deletion are indicated. 6D reveals the Southern analysis that confirms the structure of the alleles in the wildtype (wt), double-targeted (dt) and deletion (dt) clones. The deletion-specific junction fragments are indicated by the arrows.

Figure 7: Schematic representation of a provirus structure, containing *hprt*$_{\Delta}$*5'* minigene cassette, a *loxP* site, and a puromycin resistance gene, for use as a vector in one embodiment of the present invention. This particular vector would insert the *loxP* site at the 5' end of the chromosome.

Figure 8: A 3' anchor library that contains the expression cassette 3' *hprt*, puromycin resistance gene, and k14-agouti gene.

Figure 9: A 5' anchor library that contains the expression cassettes 5' *hprt*, neomycin resistance gene, and tyrosinase gene.

Figure 10: Map of an exemplary 5' endpoint targeting vector automatically excised out of a phage clone isolated from the 5' anchor library.

Figure 11: Map of an exemplary 3' endpoint targeting vector automatically excised out of a phage clone isolated from the 3' anchor library.

Figure 12: Map of pG12WT (Wildtype 3' *hprt* cassette plasmid for making chromosomal rearrangements). The sequence is identical to pG12 except that the mutation in 3' *hprt* has been fixed.

Figure 13: Map of a portion of the mouse chromosome 11 showing the general composition of the selection cassettes positioned at the chromosome endpoints, and the position of the Cre-induced deletion interval, E_2DH-D11Mit199.

Figure 14: Map of a portion of the mouse chromosome 11 showing the general composition of the selection cassettes positioned at the chromosome endpoints, and the position of the Cre-induced deletion interval, E_2DH-D11Mit69.

Detailed Description of the Invention

It will be apparent readily to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

As used herein, the term "chromosome engineering" means creating chromosome inversions, duplications or deletions.

As used herein, the term "chromosome deficiency" means a lack of a chromosome or portion of a chromosome.

As used herein, the term "chromosome inversion" means reversal of a part of a chromosome so that the genes within that part are in reverse order.

As used herein, the term "chromosome duplication" means an extra, duplicate chromosome or part of a chromosome.

As used herein, the term "ES cell" stands for embryonic stem cells: cells which are derived from early mouse embryos that can be maintained in an undifferentiated state, but, upon return to the environment of the early embryo, can contribute to all types of cells in the resulting chimera.

As used herein, the term "Cre-induced recombination" means catalysis of both intramolecular and intermolecular recombination by the Cre protein. The Cre protein is a 38 kD protein that recombines DNA between specific, 34 bp sequences called *loxP* sites.

As used herein, the term "interchromosomal recombination" means recombination between different chromosomes.

As used herein, the term "intrachromosomal recombination" means recombination between regions on the same chromosome.

As used herein, the term "*HoxB*" refers to a specific gene cluster having the same physical distance as a P1 phage and having known structure and orientation.

As used herein, the term "*Hoxb-9*" refers to a specific gene in the *HoxB* gene cluster.

As used herein, the term "*Hoxb-1*" means the 3'-most gene of the *HoxB* gene cluster.

As used herein, the term "*hprt*" means hypoxanthine phosphoribosyltransferase.

As used herein, the term "*loxP* site" means the specific 34 bp sequence recognized by Cre recombinase.

As used herein, the term "G418 resistance" means having a gene which confers resistance to G418.

As used herein, the term "*neo* resistance" means having a gene which confers resistance to G418.

As used herein, the term "puromycin resistance" means having a gene which confers resistance to puromycin.

As used herein, the term "HAT resistance" means cells resistant to media containing hypoxanthine, aminopterin and thymine. Only cells expressing the Hypoxanthine phosphoribosyl transferase (*Hprt*) and thymidine kinase genes will grow in HAT medium.

As used herein, the term "targeting deletion" means a planned deletion created by targeting an area for recombination by insertion of a *loxP* or other recombination site.

As used herein, the term "germ line transmission" refers to a chimeric animal capable of transmitting a particular trait to its offspring.

As used herein, the term "hemizygous" means genes present only once in a genotype.

As used herein, the term "heterozygous" refers to the state of an organism having two different alleles at a given locus on homologous chromosomes.

As used herein, the term "homozygous" refers to the state of an organism having the same two alleles at a given locus on homologous chromosomes.

As used herein, the term "*Gastrin*" locus means a gene on mouse chromosome 11 which encodes a peptide involved in stimulating acid secretion in the stomach. See Fuller et al., *Molec Endocrinol.* 1:306-11 (1987).

As used herein, the term "*E2DH*" locus, also known as 17HSD, is a gene whose product is involved in steroid biosynthesis. This dehydrogenase converts estrone to estradiol. See The et al., *Molec. Endocrinol.* 3:1301-06 (1989).

As used herein, the term "*Wnt3*" locus is a gene which encodes a member of the *wnt* family of growth factors. *Wnt3* is a target for activation by MMTV which causes mammary tumors. Roelink et al., *PNAS* 87:4519 (1990).

As used herein, the term "selected region" refers to that particular region of the chromosome targeted for manipulation (i.e., deletion, inversion, duplication).

In one embodiment of the present invention, a method is disclosed and claimed for deleting a selected region of genetic material in cells comprising the

steps of: inserting a first selection cassette at a 5' end of said selected region using conventional gene targeting methods, said first selection cassette comprising a first selectable marker, a first *loxP* recombination site, and a first portion of a second selectable marker; selecting cells expressing said first selectable marker; inserting a second selection cassette at a 3' end of said selected region using conventional gene targeting methods, said second selection cassette comprising a third selectable marker, a second *loxP* recombination site, and a remaining portion of said second selectable marker; selecting cells expressing said third selectable marker; expressing Cre recombinase to produce recombination between said first and second *loxP* sites; and selecting cells expressing said second selectable marker.

In particularly preferred embodiments of the present invention, the method referred to above uses a first selectable marker, a puromycin resistance gene, said second selectable marker is an *Hprt* gene, and said third selectable marker is a neomycin resistance gene.

In another particularly preferred embodiment, the first selectable marker is a puromycin resistance gene. In yet another particularly preferred embodiment, the second selectable marker is a functional *Hprt* gene. In still another preferred embodiment, the third selectable marker is a neomycin resistance gene. In another preferred embodiment, the cells are embryonic stem cells. In still another preferred embodiment, the cells are embryonic stem cells, and said cells develop into mice. In still another preferred embodiment, the cells are embryonic stem cells, and said cells are maintained as cell lines. In another embodiment of the present invention, Cre is transiently expressed Cre. In other embodiments, it is expressed either inducibly or constitutively.

In a second general embodiment of the present invention, a method is disclosed and claimed for deleting a selected region of genetic material in cells comprising the steps of: inserting a first selection cassette at a 5' end of said selected region using either conventional targeting methods or a viral vector, said first selection cassette comprising a first selectable marker, a first *loxP* recombination site, and a first portion of a second selectable marker; selecting cells expressing said first selectable marker; inserting a second selection cassette at a 3' end of said selected region using conventional gene targeting methods or

a viral vector, said second selection cassette comprising a third selectable marker, a second *loxP* recombination site, and a remaining portion of said second selectable marker; selecting cells expressing said third selectable marker; expressing transiently Cre recombinase to produce recombination between said first and second *loxP* sites; and selecting cells expressing said second selectable marker.

In one particularly preferred embodiment, the viral vector is a retrovirus. In yet another particularly preferred embodiment, the viral vector has a provirus structure comprising a cassette in turn comprising an *hprtΔ5'* cassette, a *loxP* site, and a puromycin resistance gene. In yet another particularly preferred embodiment, the viral vector has a provirus structure comprising a cassette in turn comprising an *hprtΔ5'* cassette, a *loxP* site, and a neomycin resistance gene. In still another particularly preferred embodiment, the targeting or viral vectors are a first vector for inserting said first native sequence of DNA at said 5' end, comprising: a genomic insert cloned into the vector of about 7.5 kb; a tyrosinase minigene; a Neo^r gene; a 5' *hprt* fragment; and a *loxP* site embedded into said *hprt* fragment; and a second vector for inserting said second native sequence of DNA at said 3' end, comprising: a genomic insert cloned into the vector of about 8.5 kb; a K14-Agouti gene; a $Puro^r$ gene; a 3' *hprt* fragment; and a *loxP* site embedded into said *hprt* fragment.

In a third general embodiment of the present invention, a replacement vector system, is disclosed and claimed comprising: a first vector for inserting said first native sequence of DNA at said 5' end, comprising: a genomic insert cloned into the vector of about 7.5 kb; a tyrosinase minigene; a Neo^r gene; a 5' *hprt* fragment; and a *loxP* site embedded into said *hprt* fragment; and a second vector for inserting said second native sequence of DNA at said 3' end, comprising: a genomic insert cloned into the vector of about 8.5 kb; a K14-Agouti gene; a $Puro^r$ gene; a 3' *hprt* fragment; and a *loxP* site embedded into said *hprt* fragment.

In a fourth general embodiment of the present invention, there is disclosed and claimed a method for creating defined chromosomal deficiencies, deletions, and duplications comprising the steps of: identifying a desired region of a chromosome of interest to be deleted; inserting two native sequences at each

endpoint of said region of said chromosome of interest using a first and a second targeting vector, each comprised of one or more selectable markers and a *loxP* site and an *hprt* fragment; transiently expressing Cre recombinase to produce recombination between each of two said *loxP* sites; whereby upon chromosomal rearrangement induced by said Cre recombinase, a functional *Hprt* expression cassette is reconstructed.

Other and further embodiments, features and advantages will be apparent and the invention more readily understood from a reading of the following Examples and by reference to the accompanying drawings forming a part thereof, wherein the examples of the presently preferred embodiments of the invention are given for the purposes of disclosure.

Example A:
General Strategies

The various chromosomal rearrangements described herein are designed with strong positive selection for the desired chromosomal change. Very generally, this was accomplished by targeting consecutively complementary, overlapping but non-functional *hprt-loxP* expression cassettes to the endpoints of a chromosomal interval. Cre expression (either transiently, inducibly, or constitutively) in these double-targeted ES cells induces *loxP* recombination resulting in chromosomal rearrangements specific to the relative orientation of the *loxP* sites. Since the *loxP* sites are imbedded in the *hprt* minigene fragments, the chromosomal rearrangement will also reconstruct a functional *hprt* expression cassette, therefore facilitating direct positive selection for the clones with these alterations.

The use of mouse ES (embryonic stem) cells is preferable, though not required, to execute the method the present invention. Use of these cells would, of course, allow large-scale chromosome manipulation to be introduced into a germ line, which would in turn facilitate enhanced functional study of the mouse genome.

The *loxP* sites were introduced by conventional gene targeting protocols or by viral vectors into the endpoints of the region which was to be rearranged. Or, one endpoint can be introduced by conventional methods, and the other

introduced by a viral vector. To maximize the ability to select for the rare ES cell clones in which Cre expression had successfully induced recombination between *loxP* sites, the individual *loxP* sites targeted to the endpoints of the chromosomal rearrangement were imbedded in two complementary but non-functional fragments of an *Hprt* minigene cassette. Recombination between the *loxP* sites would restore the activity of this cassette, facilitating the direct selection in HAT media of only those recombinant ES cells with the desired chromosomal structure (**Figure 1 A and B**).

In one particularly preferred embodiment of the present invention, the complementary recombination/selection substrates consist of overlapping, but incomplete, pieces of an *Hprt* minigene with a *loxP* site in the intron. These minigene fragments are linked to different positive selection cassettes which are required for selection during gene targeting. The 5′ fragment of the *loxP-Hprt* minigene is linked to a neomycin resistance gene (*hprt*$_{\Delta}$*3′* cassette), while the 3′ fragment is linked to a puromycin resistance gene (*hprt*$_{\Delta}$*5′* cassette). Cre-induced recombination between the *loxP* sites generates a fully-functional *Hprt* minigene which provides resistance to HAT selection in *Hprt*-deficient cells. The positive selectable markers are positioned so that following recombination, they are lost from the deleted chromosome. All of the clones that survive selection have the desired chromosomal structure. A similar positive selection system for detecting a chromosomal translocation has recently been reported by Smith et al., *Nature Genetics* 9:376-385 (1995). In addition, genes such as K14-agouti and tyrosinase genes can be preferably inserted into the vectors for use as color-coat markers, to aid in selecting the members of the population for which the chromosomal insert was successful. Albino mice lack the tyrosinase gene, so reinsertion of that gene is manifest by black mice in a population of white mice. Similarly, the k14-agouti gene gives yellow color to the tips of the coat hairs against a black background (i.e., it makes black mice appear brown).

Initially, a small deletion (90 kb) was constructed which encompasses the *HoxB* locus since the gene order and orientation was known. Subsequently, much larger chromosomal alterations were generated. For the latter alterations, knowledge of the transcriptional direction of the genes which serve as the rearrangement endpoints was not available. Consequently, it was necessary to

generate ES cell lines with the four possible configurations of the *hprt* minigene fragments. Because the transcriptional direction of the genes relative to the centromere was also unknown, it was not possible to predict which combination of orientations would give a deletion; however, clones with deletions are readily distinguished in culture from the clones with other classes of recombinant chromosomes because in addition to becoming HAT resistant, both of the positive selection markers are lost. The generation of a deletion reveals the relative transcriptional direction of the two deletion endpoints, and if the proximal-distal map positions are known (which was the case in these experiments), further deletions from the same endpoint are greatly simplified.

The frequency of recombination between the *loxP* sites when they were on the same chromosome varied from 6 $x10^{-7}$ to 5 $x10^{-6}$, but a direct relationship between the distance and the frequency was not apparent. The frequency of recombination was, however, significantly lower than those reported when the *loxP* sites are a few kb apart; *see*, Gu, et al., *Cell* 73:1155-64 (1993), verifying that selection is required to isolate these clones. These frequencies are derived by the transient transfection of Cre in ES cells, but might be higher under conditions of constitutive expression of Cre, for example, in a specific lineage in a transgenic mouse. The frequency of recombination was reduced by one to two orders of magnitude when the *loxP* sites were integrated in *trans* compared to the *cis* configuration. This is consistent with the knowledge that individual chromosomes occupy discrete, non-overlapping domains in an interphase nucleus.

HAT-resistant clones derived from the *trans* configuration of the double-targeted clones oriented to give deletion products were not expected to become G418 or *puro* sensitive. But approximately half of the HAT-resistant clones segregated the *puro* cassette while all retained the *neo* cassette. This segregation pattern is consistent with inter-sister-chromatid recombination (*see* **Figure 4**). Although the number of clones with the *trans* configuration was relatively small, the equal ratio of *puro+neo* to *neo*-only segregants suggests that G_2 recombination is the predominant pathway used in this case. The rescue of *hprt* negative daughter cells by metabolic cooperation also suggests that a substantial fraction of the HAT-resistant clones derived from the *cis* double-targeted clones may have been generated by the sister-chromatid pathway.

The correlation of the induced chromosomal rearrangements with the orientation of the vectors has revealed physical mapping information in this region of mouse chromosome 11. For instance, the genes described in the following Examples, *Gastrin, E2DH*, *Wnt* 3 and the *Hox B* cluster, are all transcribed in the centromere-to-telomere direction. The *E2DH-HoxB* deletion has shown that the *HoxB* cluster is oriented with the *Hoxb-9* gene nearest to the centromere.

Example B:
Deletion and duplication of 90 kb containing the HoxB cluster

The *HoxB* cluster provides an excellent substrate for the deletion strategy since the cluster is about the same physical distance as a P1 phage and the structure and orientation of the individual genes is known. *See*, Rubock, et al., *PNAS USA* 87:4751-55 (1990). Moreover, a deletion allele of *HoxB* is very useful for detailed genetic analysis of this region.

To generate a *HoxB* deletion allele, the strategy outlined in **Figure 1** was followed. The *hprt*Δ3′ cassette was used to construct a targeting vector for *Hoxb-1,* the most 3′ gene of the cluster, and targeted clones were identified (**Figure 2**). An ES clone with the *Hoxb-1* targeted allele (**Figure 2B**) was expanded and transfected with the *Hoxb-9* targeting vector containing the complementary *hprt*Δ5′ cassette. The minigene fragments were oriented in the targeting vectors so that, after targeting, they would be in the correct order and orientation with the positive selection cassettes (*neo* and *puro*) located between the *loxP* sites (**Figure 2C**). Double-targeted clones were identified (**Figure 2C**), and half of these clones would be expected to have both targeted alleles on the same chromosome (*cis*) and half should have the targeted alleles on different homologues (*trans*).

To induce the recombination between the *loxP* sites, several independent double-targeted (*Hoxb-1* and *Hoxb-9*) clones were expanded and transiently transfected with a Cre expression cassette and placed under HAT selection. Control transfections without Cre did not yield any HAT-resistant clones. What follows is a more detailed description of the method employed in this example.

As depicted in **Figur 1A**, the PGK*Hprt* minigene was modified by the insertion of a *loxP* site from pBS64 (*HindIII-EcoRI*, Klenow blunt) into the unique *XbaI* site (Klenow blunt) in the *hprt* intron. Insertion of the *loxP* site did not disrupt the cassette's HAT resistance function (*not shown*). The *loxP-Hprt* cassette was divided into two overlapping pieces: *hprt*Δ*3'*, which contains the PGK promoter, the *hprt* exons 1 and 2, the *loxP*-intron, *hprt* exons 3-6, and the SV40 poly A signal. In **Figure 1A**, *hprt*Δ*5'* and *hprt*Δ*3'* have a 2kb overlap including the *loxP* site; independently, *hprt*Δ*5'* and *hprt*Δ*3'* do not provide HAT resistance, but they do when co-electroporated. *Hprt*Δ*5'* and *hprt*Δ*3'* were ligated to positively selectable cassettes (*hprt*Δ*3'* to the *pol II neo* gene and *hprt*Δ*5'* to a PGK-puromycin resistance gene); in both cases, the positive markers replaced the deleted part of the *loxP-Hprt* cassette, ensuring that, upon recombination, they are separated from the reconstituted cassette. **Figure 1C** depicts the general strategy for making deletions consisting of 3 steps: Step 1: conventional replacement-type gene targeting used to replace the *Hoxb*-1 gene with the *hprt*Δ*3'-neo* cassette; Step 2: ES cells identified as correctly targeted are used as a substrate to insert the *hprt*Δ*5'-puro* cassette into the endogenous *Hoxb*-9 gene by conventional replacement-style targeting (the *cis* configuration is illustrated here); and Step 3: transient expression of Cre induces recombination between the *loxP* sites which reconstructs a functional *hprt* minigene. In **Figure 1 C4**, the intra-chromosomal recombination pathway is illustrated, and in **Figure 1 C5**, cells with the recombinant (deleted) chromosome to be positively selected in HAT media and a chromosomal ring are generated by the intra-chromosomal pathway. This is believed to be unstable and lost during the growth of the colony.

The targeting vector for *Hoxb-1* consists of a 3.5 kb *BglII-NcoI* fragment (5' homologous arm); the *Hoxb-1* coding sequence (1.7 kb *Nco-BglII*) was replaced by the *hprt*Δ*3'-neo* cassette and a 2 kb *BglII-PvuII* fragment (3' homologous arm). The vector was linearized with *SalI* and a 10 μg was electroporated into *hprt*-negative AB2.2 ES cells. G418 selection was applied 24 hours after the electroporation and resistant clones were arrayed in 96 well plates, and targeted clones were detected by Southern analysis. A single targeted clone out of 384 clones analyzed was identified with the predicted structure of

the targeted allele using probes 5′ and 3′ of the *Hoxb-1* gene. This clone was expanded and transfected with the *Hoxb-9* targeting vector. The targeting vector for *Hoxb-9* consisted of a 6.2 kb HindIII fragment of homology which included exon 1. The *hprtΔ5′-puro* cassette was cloned into the unique *SalI* site in exon 1. The orientation of the cassette was such that, when targeted, the *loxP* sites in the *hprtΔ* cassettes would be in the same orientation. The vector was linearized and 10 μg of vector was electroporated into clone #298 AB2.2 cells and plated on SNLP (*puro* resistant SNL76/7 cells). Puromycin selection (5 μgml) was applied 24 hours after electroporation. Resistant clones were arrayed in 96 well plates and screened for targeted clones by Southern analysis. Double-targeted clones were detected at a frequency of 6%. Multiple independent double-targeted clones were expanded and independently transiently transfected (by electroporation) with 20μg of a supercoiled Cre expression cassette pOG231. HAT selection was applied 48 hours after the electroporation. HAT-resistant clones were arrayed and analyzed by Southern Blot analysis.

As evidenced by **Table 1**, two classes of double-targeted clones could be distinguished by this assay.

TABLE 1

Interval	Distance	Frequency (10^{-7}) Deletion I	Deletion II	Inversion
Hoxb9 - Hoxb1	90kb	153	0.5	ND
Hoxb9 - E2DH	3-4cM	6	1	43
Gastrin - E2DH	1 Mb	470	1.8	334
E2DH - Wnt3	3-4cM	30	4	19

Table 1 reports the frequency of Cre-mediated recombination as a function of distance between the *loxP* sites. Deletion frequencies are illustrated for both Class I and Class II clones while inversion frequencies are only illustrated for Class I clones.

One class (Type I) yielded HAT-resistant recombinants at frequencies averaging 1 x 10^{-6} per treated cell, while a second class (Type II) yielded HAT-resistant clones at a much lower frequency.

Table 2 shows the frequency of Cre-mediated recombination as a function of distance between the *loxP* sites.

TABLE 2

Example B

RECOMBINATION FREQUENCY ON THE MOUSE CHROMOSOME 11

Interval	Deletion		Inversion		Duplication	
	Cis	Trans	Cis	Trans	Cis	Trans
Gastrin-E_2DH (1 Megabase)	476	1	355	0	166	2
E_2DH-D11MIT199 (2 cM)	102	3	293	0	N/A	N/A
HoxB-E_2DH (3-4 cM)	2	0	43	0	N/A	N/A
E_2DH-Wnt3 (3-4 cM)	36	4	19	0	N/A	N/A
E_2DH-D11MIT69 (22 cM)	0	0	3	0	N/A	N/A

(1 X 10^7 ES cells were electroporated with 20 ug pOG 231 (Cre expression plasmid) and were selected with HAT medium.)

Table 2 is similar to **Tabl 1**, except that the former shows an additional deletion frequency for an additional interval (E_2DH-D11Mit69); it also shows duplication frequency; and finally it shows additionally deletion, inversion, and duplication frequencies for **both** *cis-* and *trans-*.

Example C:
Cis and trans recombination

Two types of clones might correspond to the *cis* or *trans* configuration of the *loxP* sites. The HAT-resistant clones derived at a high frequency from Type I clones might be products in intrachromosomal recombination or sister chromatid exchange (*loxP* sites in *cis*). Type II clones might require interchromosomal or inter-sister-chromatid recombination between homologous chromosomes (*loxP* sites in *trans*), which may occur relatively infrequently. These different pathways were distinguished by analyzing the markers in recombinant HAT-resistant clones.

Deletion of the *HoxB* cluster from a chromosome double targeted in *cis* would be accompanied by the loss of the *neo* and *puro* cassettes. These are either segregated (sister-chromatid pathway) or a ring is formed which is presumed to be unstable (**Figure** 1). The loss of both markers could be documented in most of the HAT-resistant clones derived from Type I double-targeted clones, consistent with the hypothesized *cis* configuration of the Type I clones.

As anticipated, the consecutive targeting events and the Cre-induced recombination event results in the formation of novel restriction fragments (**Figure 2**). External probes identify the novel junction fragments using an *NheI* digest since there is not an *NheI* site present in the *loxP-hprt* cassette. An internal probe confirms the loss of 90kb of sequence by dosage difference between the wildtype and the deletion clones (**Figures 2 E and F**).

Example D:
E2DH-Gastrin 1Mb deletion

One of the goals behind the methods of the present invention is to construct chromosomal deletions so that regions of the genome can be tested for tumor suppressor activity. Many candidate regions have been identified from

loss of heterozygosity (LOH) studies. One well-defined region in human breast cancer maps close to the *Gastrin* locus on human chromosome 17q close to BRCA1. Miki, et al., *Science* 266:66-71 (1994). This putative sporadic tumor suppressor locus maps in a conserved linkage group on mouse chromosome 11 between *Gastrin* and the *E2DH* locus. The generation of hemizygous mice with a deficiency that encompasses this locus functionally tests if this region contains a sporadic breast cancer gene that is involved in mammary neoplasia.

The large size of the regions which contain putative sporadic tumor suppressor loci complicates substantially the use of deletion strategy. In the absence of a YAC cloning contig which spans the relevant genetic interval, the gene order and orientations were not known. This is an important consideration since both the order and the orientation of the *Hprt* minigene fragments will determine the type of chromosomal rearrangement that is required to reconstruct a functional *Hprt* cassette. The possible orientations are illustrated in **Figure 1D.** The recombinant chromosomes include deletions, duplications, inversions, and di- and acentric chromosomes (**Figure 1E**). These rearranged chromosomes can be distinguished on Southern blots by the appearance of novel junction fragments, but the most rapid identification of the clones with deletions can be obtained from selection using neomycin and puromycin resistance cassettes which have been configured to lie between the *loxP* sites in the to-be-deleted interval (see **Figure** 1).

To construct ES cell lines with large deletions between the *Gastrin* and the *E2DH* locus (containing SBC I) in the absence of *a priori* knowledge of the gene order and orientation, all four possible arrangements of the *hprt* minigene fragments were made and tested, only one of which will generate deletions. Two targeting vectors were constructed for each deletion endpoint representing the two possible orientations of the *hprt* minigenes. The *hprt*Δ*3'* cassette was targeted to the *E2DH* locus with the alternative minigene orientations (A or B). Targeted clones representing both the A and B orientations were, in turn, transfected with the targeting vectors representing the different orientations of the *hprt*Δ*5'* cassette (A or B) at the *Gastrin* locus. Multiple independent, targeted clones were isolated representing the four different minigene configurations to ensure that clones with the *cis* and the *trans* configurations

were likely to be represented. Each of these clones was expanded, transfected with a Cre expression cassette and plated under HAT selection. What follows is a more detailed description of the method employed in this example.

Overlapping λ phage containing the mouse *E2DH* locus were isolated from a mouse 129Sv/Ev genomic library using a human *E2DH* cDNA probe. Unlike the duplicated human *E2DH* locus, the mouse locus is present at a single copy. λ phage containing the mouse *Gastrin* locus were isolated from the same library using a PCR fragment from the rat *Gastrin* cDNA. The mouse *E2DH* gene and *Gastrin* genes had not been mapped; to confirm that these genes mapped to mouse chromosome 11, a hamster/mouse hybrid cell line in which the mouse chromosome 11 is the only mouse genetic material was hybridized to probes specific for the mouse *Gastrin* and *E2DH* loci.

Standard gene replacement targeting vectors were constructed from these genomic clones. *E2DH* vector: a total of 8.0 kb of homology was used. The *XhoI-XbaI* 5.5 kb fragment containing the entire *E2DH* coding sequence was replaced with the *hprt*$_{\Delta}$*3′* minigene cassette in both orientations. In the *Gastrin* vector, a total of 7.5 kb of homology was used. The 3.5 kb *XhoI-NheI* fragment containing the *Gastrin* coding region was replaced with the *hprt*$_{\Delta}$*5′* cassette in both orientations.

The two vectors were separately transfected into AB2.2 ES cells. G418 resistant clones were obtained for each vector. Clones were gridded onto 96 well plates and screened for targeted clones. Targeted clones were identified at a ratio of 1/25 for the A orientation vector and 1/25 for the B orientation vector. The two types of targeted ES cells were assayed for totipotency by generating chimeras which tested for germ line transmission. Totipotent *E2DH*-targeted ES cell lines were identified for both the A and the B orientation and these were transfected with the vectors which target the *Gastrin* locus. Puromycin resistant clones were arrayed on 96 well plates and screened for targeted clones. All four classes of double targeted clones were obtained. For simplicity, this figure only shows the double targeted ES cell having the *hprt*$_{\Delta}$*5′* and *hprt*$_{\Delta}$*3′* cassettes in the A orientation and in *cis*.

The double targeted ES cell clones were transfected with the Cre expression plasmid as previously described. HAT^r colonies were recovered and

sibselected to test for puromycin and G418 resistance. Individual clones were expanded and analyzed for junction fragments using multiple probes. Each blot was hybridized with two probes, one from the *E2DH* locus and the other from the *Gastrin* locus. The frequency of obtaining HAT resistant colonies from the different clones is summarized in **Table 3**.

TABLE 3

Category	Class	Clones tested	m HAT^r (10^7)	G418	Puro
AA	I II	4 2	470 1	S R	S R/S
AB	I II	5 1	344 0	R -	R -
BA	I II	3 2	377 0	R -	R -
BB	I II	3 6	166 1.8	R R/S	R R

Table 3 reveals the frequency of the Cre-mediated recombination and retention of the markers in recombinant clones. All of the data is derived from the *E2DH-Gastrin* double targeted clones. The categories of clones are illustrated in **Figure 1D**, and the expected products are described in **Figure 1E**. Class I double-targeted clones give a high frequency of HAT-resistant recombinants, while Class II clones give a low frequency of HAT-resistant clones. Retrospective analysis has revealed that the class I clones and Class II clones have the targeted genes in *cis* and *trans*. S and R refer to resistance or sensitivity to G418 or puromycin as assayed by selection. Both resistant and sensitive clones were recovered.

HAT-resistant clones were recovered from each of the four alternative split minigene configurations. The individual clones within a specific orientation

group could be placed into one of two classes, based on the frequency with which HAT-resistant clones could be recovered (**Table 3**). Selection analysis identified the AA class I HAT-resistant clones as those that had lost the neomycin and puromycin resistance genes; these clones are the most likely to have the desired deletion. Since the AA class I clones gave the deletion product, this allows predictions to be made on the likely products of the alternative configurations: BB gives duplications, and AB or BA should give inversions. These predictions have been confirmed by detailed molecular analysis summarized in **Figure 3**. In particular, the juxtaposition of the *hprt* minigene fragments which were previously positioned approximately 1Mb apart in the genome results in unique junction fragments that are specific for the different types of rearrangement.

The AA type II clones yield HAT-resistant recombinants at a low frequency. It was hypothesized that these clones represented the cases where the deletion selection cassettes had integrated in *trans*. Interchromosomal recombination would result in both the *neo* and *puro* resistance genes being located on one chromosome, while the reconstructed *hprt* minigene would be on the homologue. Thus it would be anticipated that all of the positive selection markers would be retained in such a cell. Sibselection identified two classes of HAT-resistant clones which were represented at approximately equal frequencies. One type only retained the *neo* cassette, and a second type retained both the *neo* and the *puromycin* resistance cassettes (Table 1A). The segregation of the *puro* resistance gene from the *neo* cassette is explained readily if Cre-induced recombination between sister chromatids (**Figure 4**) occurred. This occurrence was confirmed by the molecular analysis of these clones. While the clones with the duplicated and deleted chromosomes can be generated by either interchromosomal or non-sister chromatid exchange, the clones which only carry the *neo* cassette can only have arisen by the non-sister chromatid recombination pathway. These clones have been confirmed to carry both the deletion chromosome and the non-recombinant chromosome with only the *E2DH* targeted locus (**Figure 4**).

The clones with the deletion on one chromosome and the duplication on the other are genetically balanced. Therefor these clones were considered to be the best candidates for germ line transmission. Four independent clones were

injected into blastocysts, representing clones descended from both the A and B orientation of the *E2DH* targeted allele. Alleles from three of these clones were transmitted into the germ line, despite three cycles of subcloning and expansion. Segregation of the deletion and duplication alleles from a chimeric male is illustrated by gene dosage analysis (**Figure 5**). Mice which are hemizygous for this deletion (1 copy) are fully viable. Mice which are heterozygous for the duplication (3 copies) or homozygous (4 copies) are also fully viable (**Figure 5**) and fertile.

Example E:

Deletion of two 3-4 centimorgan regions on chromosome 11

Given the apparent insensitivity of the Cre-induced recombination to the distance between the *loxP-hprt* substrates, two additional experiments were performed to investigate if Cre could delete a larger fragment. Two 3-4cM intervals were chosen, proximal or distal to the *E2DH* locus on mouse chromosome 11. This region is syntectic with a region on human chromosome 17q where loss of heterozygosity studies have identified several distinct regions that are likely to contain tumor suppressor genes which are mutated in 30-70% of sporadic breast cancer. These regions have been termed SBCI, SBCII and SBCII (**Figure 6A**).

Since the *E2DH-Gastrin* deletion had revealed the orientation of the *E2DH* locus, one of the A orientation *E2DH*-targeted clones was selected for the proximal deletion and a B orientation *E2DH*-targeted clone was selected for the distal deletion. Targeting vectors (two orientations) were constructed for the *HoxB* locus (*Hoxb-9*) and for *Wnt3* (*see* Roelink, et al., *PNAS USA* 87:4519-23 (1990)). Double-targeted clones were generated, transfected with the Cre expression cassette and HAT-resistant clones were selected. One vector orientation yielded *G418* and *puro* sensitive clones which were hypothesized to have a 3-4cM deletion, while the other orientation yielded HAT-resistant clones which all retain the *neo* and *puro* cassettes. This latter category of clones were confirmed to be inversions of the 3-4cM interval by molecular analysis. The molecular analysis of the G418 and *puro* sensitive clones identified two classes of clones that occur with approximately equal frequency. The first type of clone

was consistent with a simple deletion event, illustrated for the *Hoxb9-E2DH* deletion (**Figur 6D**). The other class of clone exhibited the expected deletion junction fragment, but also retained a junction fragment that is diagnostic for the targeted chromosome, but should have been lost during the deletion event (illustrated for the *E2DH-Wnt3* deletion in **Figure 6D**). The retention of this junction fragment and the acquisition of the expected deletion-specific fragment in about half the clones can be explained by two different recombination pathways. The pure clones are believed to be products of an inter-chromosomal pathway (**Figure 1C**), while those clones that retain the primary targeted allele may reflect a sister-chromatid exchange. While the duplicated chromosome should be segregated to a daughter cell and does not carry the reconstructed *hprt* minigene, extensive metabolic co-operation between the $hprt^{+}$ and $hprt^{-}$ ES cells in a colony facilitate cross rescue and substantial contribution of the $hprt^{-}$ daughter cells to the HAT-resistant clones. What follows is a more detailed description of the method employed in this example.

E2DH and *Hoxb9* vectors have been described previously. *Wnt3* genomic clones were isolated from a 129Sv/Ev genomic library using a cDNA probe. Conventional replacement targeting vectors containing 7.0kb of homology were constructed. The *hprt*Δ*5′* cassette replaces a 2.1 fragment (contains exons 3 and 4) of the *Wnt3* gene. The *Hoxb9* vectors and the *Wnt3* vectors were independently targeted into the *E2DH* targeted cell lines. An "A" orientation clone was used for the *Hoxb9* targeting vectors. Transfected cells were selected in puromycin and targeted clones were identified as previously described. Multiple independent double targeted clones were transfected with the Cre recombinase. HAT-selection was used to isolate recombinant clones which were sib-selected and tested by Southern analysis for the predicted junction fragments.

Example F:

Using a virus rather than targeting to effect the recombination

Rather than relying on traditional targeting techniques, either or both of the desired deletion endpoints can be added to the genome by means of a retrovirus. In this example, a viral vector is used to insert the endpoint at the 5' end only. **Figure 7** is a schematic representation of a provirus structure

suitable for this use, which is comprised of an *hprtΔ5'* minicassette, a *loxP* site and a puromycin resistance gene. Hence, the provirus structure is similar to the non-viral targeting vectors described in the previous examples.

In this example, only the 5' endpoint was inserted using the viral vector depicted in **Figure 7**, though in other embodiments, both endpoints can be added using viral vectors. Chromosomal deletions were induced using the methods substantially as described in the previous examples. Hence, insertions are made at the two endpoints framing the desired chromosomal deletion. The insertions are preferably made one at a time, and involve replacing a first native sequence (i.e., the first endpoint) on the chromosome of interest with a first selection cassette. This selection cassette consists of three elements: a first selectable marker, a *loxP* site located in the *hprt* minigene intron, and a first portion of a second selectable marker, preferably a non-functional fragment of an *Hprt* minigene cassette. The first selectable marker is preferably a neomycin resistance gene (*hprtΔ5'* cassette) or a puromycin resistance cassette (*hprtΔ3'* cassette). The cells expressing the marker (either the neomycin resistance gene or the puromycin resistance gene) are then selected. Next, the process is essentially repeated for second endpoint on the chromosome of interest. Thus, the cells selected possess both *loxP* sites framing the desired portion of the chromosome to be deleted. The difference in this step is that the *hprt* minigene fragment—also non-functional—is the **complementary** portion to that inserted into the first endpoint. Third, the selected cells are contacted with Cre, which may be expressed in one of the three ways described above, which induces recombination between the *loxP* sites. This recombination generates a fully functional *Hprt* minigene. This minigene provides resistance to HAT selection in *hprt* deficient cells. Additionally, the positive selectable markers are positioned so that following recombination, they are lost from the deleted chromosome. Therefore, the methods for inducing the deletion described in this example are nearly identical to those for practicing the method using a non-viral vector. Inherent differences in method and technique that result from using a viral versus non-viral vector are well-known to the skilled artisan, hence a detailed description of any embodiment of the method of the present invention involving non-viral vectors could be easily adapted by the skilled artisan using

a viral vector. The recombination efficiencies obtained from using the viral vector (described in **Figure 7**) at the 5' endpoint are shown below in **Table 4**.

TABLE 4

cell out of 10^7 cre electroporated ES cells

	HAT^r	$Puro^r$	NO Drug	Rec Efficiency
plate #3	275	$1.62x10^5$	$3.9x10^5$	$1.7x10^{-3}$
plate #4	388	$2.54x10^5$	$3.9x10^5$	$1.5x10^{-3}$
Average	331	$2.09x10^5$	$3.9x10^5$	$1.6x10^{-3}$

Example G:

<u>Frequency of Cre-induced deletion between EDH and D11 Mit199 using an improved targeting vector</u>

This example illustrates the enhanced Cre-induced deletion frequency using a different targeting vector compared with that used in the previous examples. The details of the protocol are substantially as described in the previous examples. Details of this "improved vector" compared with the vector used in the previous examples are shown in **Figures 10** through **12** (the original vector is shown by comparison in **Figure 12**). **Figure 10** shows a map of an exemplary 5' endpoint targeting vector automatically excised out of a phage clone isolated from the 5' anchor library. The 5' anchor library is shown in **Figure 9**; this anchor library contains the expression cassettes 5' *hprt*, neomycin resistance

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gene, and tyrosinase gene. **Figure 11** shows a map of an exemplary 3' endpoint targeting vector automatically excised out of a phage clone isolated from the 3' anchor library. The 3' anchor library is shown in **Figure 8**; this anchor library contains the expression cassette 3' *hprt*, puromycin resistance gene, and k14-agouti gene. **Figur 12** shows a map of pG12WT (Wildtype 3' *hprt* cassette plasmid for making chromosomal rearrangements). At the bottom of **Figure 12**, a comparison of the sequence used to generate the data in Examples B-E versus the sequence used to generate the data in this and following examples is shown. As evidenced by **Figure 12**, the two sequences are identical to pG12 except that the mutation in 3' *hprt* of the wildtype cassette plasmid has been fixed.

Figure 13 shows the portion of the mouse chromosome 11 at which the deletion strategy is directed; **Figure 13** also shows the general composition of the selection cassettes positioned at the chromosome endpoints, and the position of the Cre-induced deletion interval, E_2DH-D11Mit199.

Table 4 shows the frequency of Cre-induced deletion between E_2DH and D11Mit199, which can be compared with **Tables 1A, 1B**, and **4**. The frequency shown is the number of HAT-resistant colonies per Cre-electroporated cell. The numbers are obtained by averaging data from at least two experiments with at least two cell lines (except for the new vector in the trans configuration). A comparison of the data presented in **Table 4** with those in **Tables** 1, 2, and 3 reveal that the cassette shown in Figure 11 mediates recombination approximately 10^3 more efficiently than the cassette used to generate the data in **Tables 1, 2**, and **4**.

Example H:

<u>Frequency of Cre-induced deletion between E_2DH and D11 Mit69 using an improved targeting vector</u>

Similar to Example H, this example also illustrates the enhanced Cre-induced deletion frequency using a different targeting vector compared with that used in the previous examples. Example H illustrates the Cre-induced deletion frequency between E_2DH and D11Mit199–a distance of about 2 CM. By contrast, Example I illustrates the Cre-induced deletion frequency between E_2DH and D11Mit69–a distance of about 22 CM). The details of the protocol are substantially as described in the previous examples. Details of this "improved

vector" compared with the vector used in the previous examples are shown in **Figures 10** through **12** (the original vector is shown by comparison in **Figure 12**). **Figure 10** shows a map of an exemplary 5' endpoint targeting vector automatically excised out of a phage clone isolated from the 5' anchor library. The 5' anchor library is shown in **Figure 9**; this anchor library contains the expression cassettes 5' *hprt*, neomycin resistance gene, and tyrosinase gene. **Figure 11** shows a map of pG12WT (Wildtype 3' *hprt* cassette plasmid for making chromosomal rearrangements). The sequence is identical to pG12 except that the mutation in 3' *hprt* has been fixed.

Figure 14 shows the portion of the mouse chromosome 11 at which the deletion strategy is directed; **Figure 14** also shows the general composition of the selection cassettes positioned at the chromosome endpoints, and the position of the Cre-induced deletion interval, E_2DH-D11Mit69. **Table 6** shows the frequency of Cre-induced deletion between E_2DH and D11Mit69, which can be compared with **Tables 1A, 1B**, and **4**.

TABLE 6

Example H

Markers	Frequency
HAT^r, $Puro^s$, Neo^s	$5.8 \pm 3.3 \times 10^{-6}$ (n=5) / 2×10^{-5} (n = 1)
HAT^r, $Puro^r$, Neo^r	$1.1 \pm 0.4 \times 10^{-5}$ (n=10) / 3×10^{-5} (n = 1)

Two frequencies are reported in each row, reflecting two separate trials. The "frequency" of Cre-induced *loxP* recombination is expressed as the number of HAT^r colonies per Cre-electroporated cell. The number of independent doubly targeted cell lines is denoted by "n=."

TABLE 5

Example G

3' *hprt* cassette	Cis	Trans
Old (mutant)	$2 \pm 0.5 \times 10^{-5}$	$6.5 \pm 3.2 \times 10^{-7}$
New (wildtype)	$2.3 \pm 1.3 \times 10^{-2}$	1.5×10^{-4}

WHAT IS CLAIMED IS:

1. A method for deleting a selected region of genetic material in cells comprising the steps of:

inserting a first selection cassette at a 5' end of said selected region using conventional gene targeting methods, said first selection cassette comprising a first selectable marker, a first *loxP* recombination site, and a first portion of a second selectable marker;

selecting cells expressing said first selectable marker;

inserting a second selection cassette at a 3' end of said selected region using conventional gene targeting methods, said second selection cassette comprising a third selectable marker, a second *loxP* recombination site, and a remaining portion of said second selectable marker;

selecting cells expressing said third selectable marker;

expressing Cre recombinase to produce recombination between said first and second *loxP* sites; and

selecting cells expressing said second selectable marker.

2. The method of Claim 1 wherein said first selectable marker is a puromycin resistance gene, said second selectable marker is an *Hprt* gene, and said third selectable marker is a neomycin resistance gene.

3. The method of Claim 1 wherein said first selectable marker is a puromycin resistance gene.

4. The method of Claim 1 wherein said second selectable marker is a functional *Hprt* gene.

5. The method of Claim 1 wherein said third selectable marker is a neomycin resistance gene.

6. The method of Claim 1 or 2 wherein said cells are embryonic stem cells.

7. The method of Claim 1 or 2 wherein said cells are embryonic stem cells, and said cells develop into mice.

8. Th method of Claim 1 or 2 wherein said cells are embryonic stem cells, and said cells are maintained as cell lines.

9. The method of Claim 1 or 2 wherein said Cre is transiently expressed Cre.

10. The method of Claim 1 or 2 wherein said Cre is inducibly expressed Cre.

11. The method of Claim 1 or 2 wherein said Cre is constitutively expressed Cre.

12. A method of deleting a selected region of genetic material in cells comprising the steps of:

inserting a first selection cassette at a 5' end of said selected region using either conventional targeting methods or a viral vector, said first selection cassette comprising a first selectable marker, a first *loxP* recombination site, and a first portion of a second selectable marker;

selecting cells expressing said first selectable marker;

inserting a second selection cassette at a 3' end of said selected region using conventional gene targeting methods or a viral vector, said second selection cassette comprising a third selectable marker, a second *loxP* recombination site, and a remaining portion of said second selectable marker;

selecting cells expressing said third selectable marker;

expressing transiently Cre recombinase to produce recombination between said first and second *loxP* sites; and

selecting cells expressing said second selectable marker.

13. The method of Claim 12 wherein the viral vector is a retrovirus.

14. The method of Claim 12 wherein the viral vector has a provirus structure comprising a cassette in turn comprising an *hprtΔ5'* cassette, a *loxP* site, and a puromycin resistance gene.

15. The method of Claim 12 wherein the viral vector has a provirus structure comprising a cassette in turn comprising an *hprtΔ5'* cassette, a *loxP* site, and a neomycin resistance gene.

16. The method of Claim 12 wherein the targeting or viral vectors are a first vector for inserting said first native sequence of DNA at said 5' end, comprising:

a genomic insert cloned into the vector of about 7.5 kb;
a tyrosinase minigene;
a neomycin resistance gene;
a 5' *hprt* fragment; and
a *loxP* site in said *hprt* fragment intron;

and a second vector for inserting said second native sequence of DNA at said 3' end, comprising:

a genomic insert cloned into the vector of about 8.5 kb;
a K14-agouti gene;
a puromycin resistance gene;
a 3' *hprt* fragment; and
a *loxP* site embedded in said *hprt* fragment intron.

17. The method of Claim 16 wherein said 3' *hprt* fragment has the following base pair sequence at the start of exon 3: GAC,TGA,ACG,TCT,TCG,AGA,TGT,GAT, GAA,GGA,GAT.

18. A replacement vector system comprising:

a first vector for inserting said first native sequence of DNA at said 5' end, comprising:

a genomic insert cloned into the vector of about 7.5 kb;

a tyrosinase minigene;

a neomycin resistance gene;

a 5' *hprt* fragment; and

a *loxP* site in said *hprt* fragment intron;

and a second vector for inserting said second native sequence of DNA at said 3' end, comprising:

a genomic insert cloned into the vector of about 8.5 kb;

a K14-agouti gene;

a puromycin resistance gene;

a 3' *hprt* fragment; and

a *loxP* site embedded in said *hprt* fragment intron.

19. The vector system of Claim 18 wherein said 3' *hprt* fragment has the following base pair sequence at the start of exon 3: GAC,TGA,ACG,TCT,TCG,AGA, TGT,GAT,GAA,GGA,GAT.

20. A method for creating defined chromosomal deficiencies, deletions, and duplications comprising the steps of:

identifying a desired region of a chromosome of interest to be deleted;

inserting two native sequences at each endpoint of said region of said chromosome of interest using a first and a second targeting vector, each comprised of one or more selectable markers and a *loxP* site and an *hprt* fragment;

transiently expressing Cre recombinase to produce recombination between each of two said *loxP* sites;

whereby upon chromosomal rearrangement induced by said Cre recombinase, a functional *Hprt* expression cassette is reconstructed.

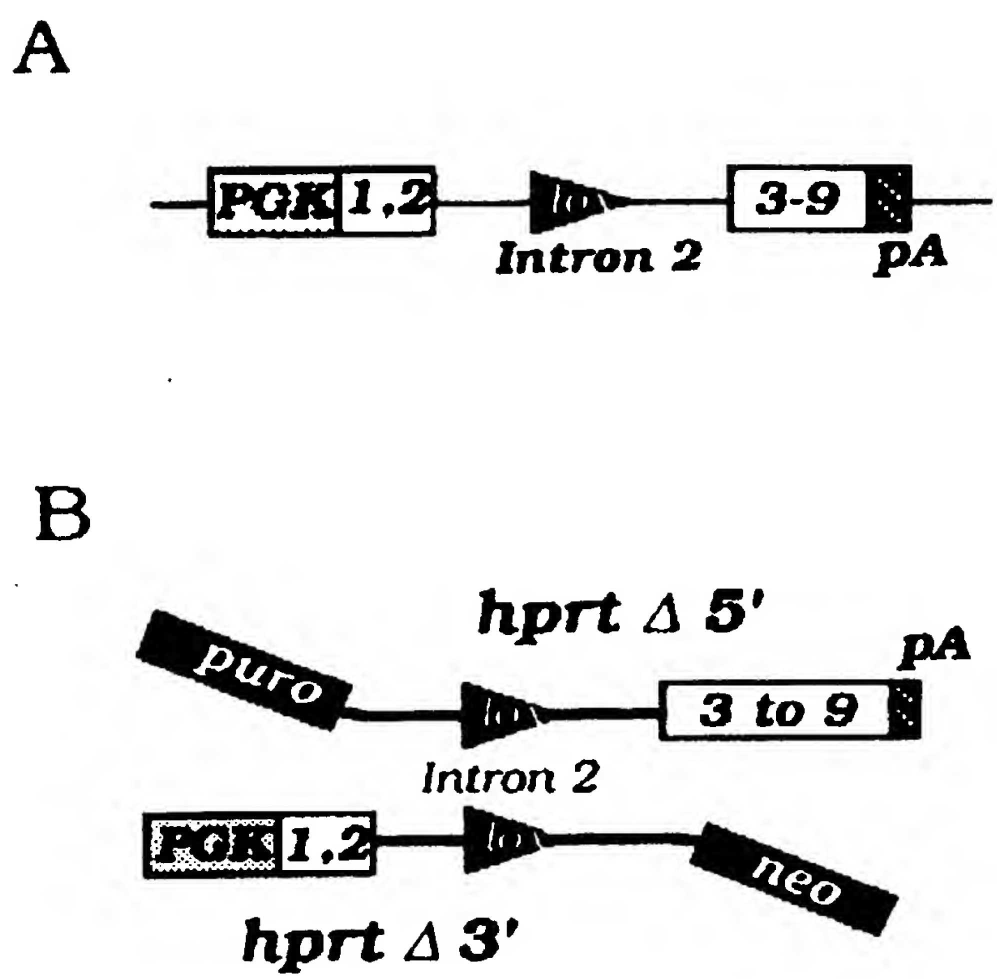
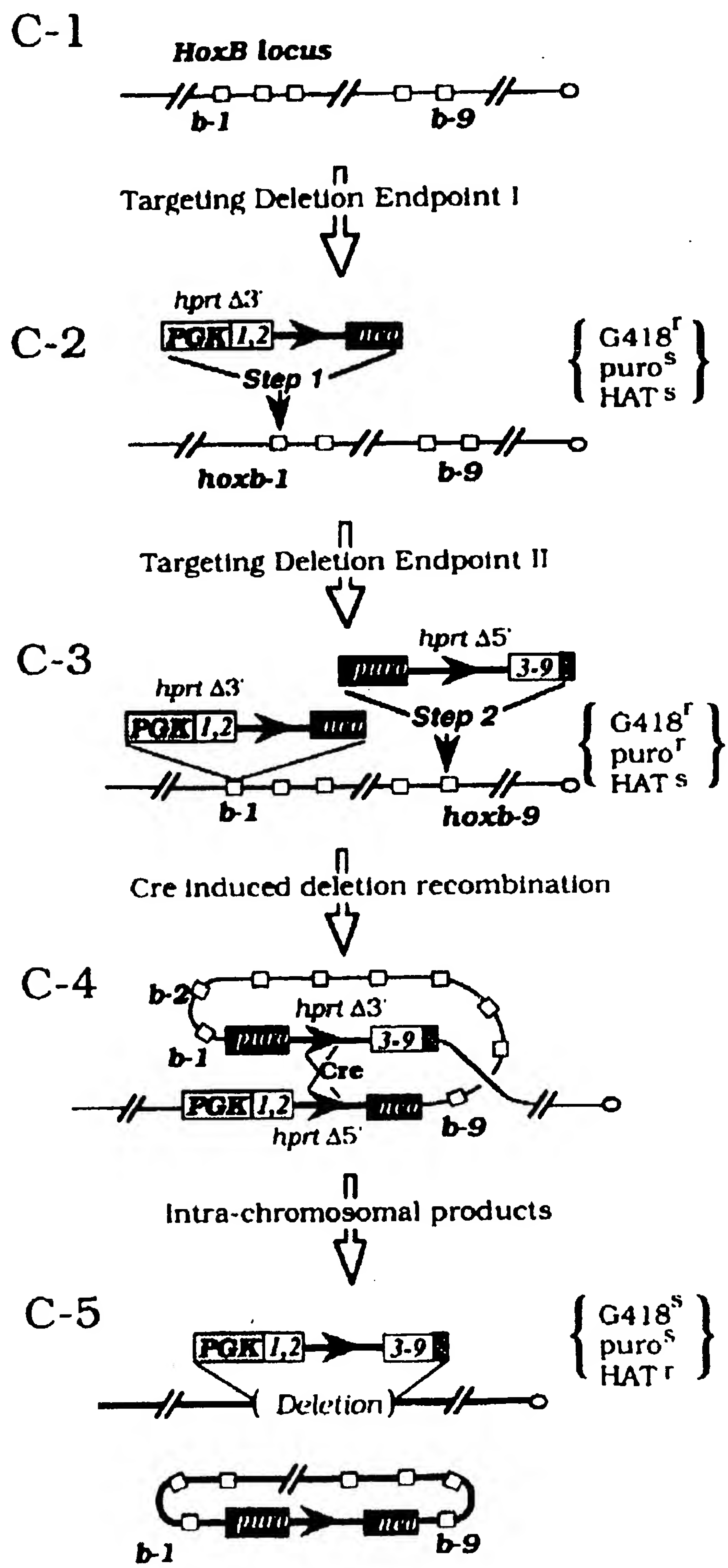
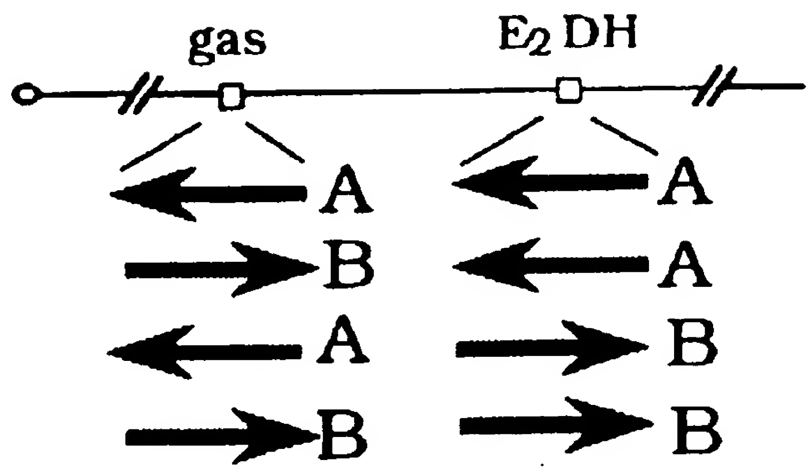

A
PGK
1,2
3-9
Intron 2
pA
B
hprt Δ 5'
pA
puro
3 to 9
Intron 2
PGK
1,2
neo
hprt Δ 3'

Figure 1


C-1
HoxB locus
b-1
b-9
Targeting Deletion Endpoint I
C-2
hprt Δ3'
PGK
1,2
neo
Step 1
hoxb-1
b-9
G418[r]
puro[s]
HAT[s]
Targeting Deletion Endpoint II
C-3
hprt Δ5'
puro
3-9
hprt Δ3'
PGK
1,2
neo
Step 2
b-1
hoxb-9
G418[r]
puro[r]
HAT[s]
Cre induced deletion recombination
C-4
b-2
hprt Δ3'
puro
3-9
b-1
Cre
PGK
1,2
neo
hprt Δ5'
b-9
Intra-chromosomal products
C-5
PGK
1,2
3-9
Deletion
G418[s]
puro[s]
HAT[r]
puro
neo
b-1
b-9

Figur 1

D



E

Orientation	Phase	HAT^R products
AA	cis	del
	trans	del + dup
BA	cis	inv
	trans	dicen + ditel
AB	cis	inv
	trans	dicen + ditel
BB	cis	dup
	trans	dup + del

Figure 1

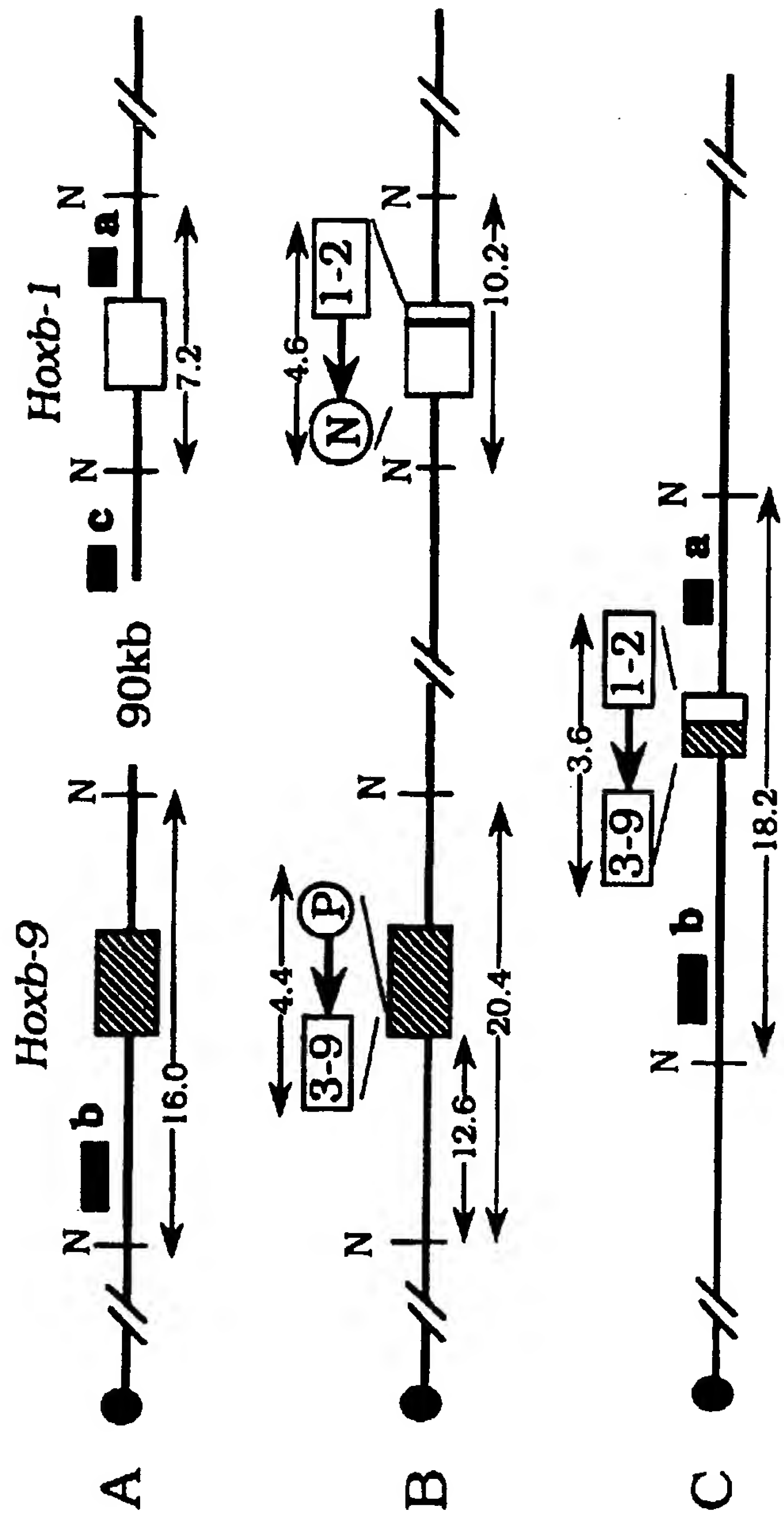


Figure 2

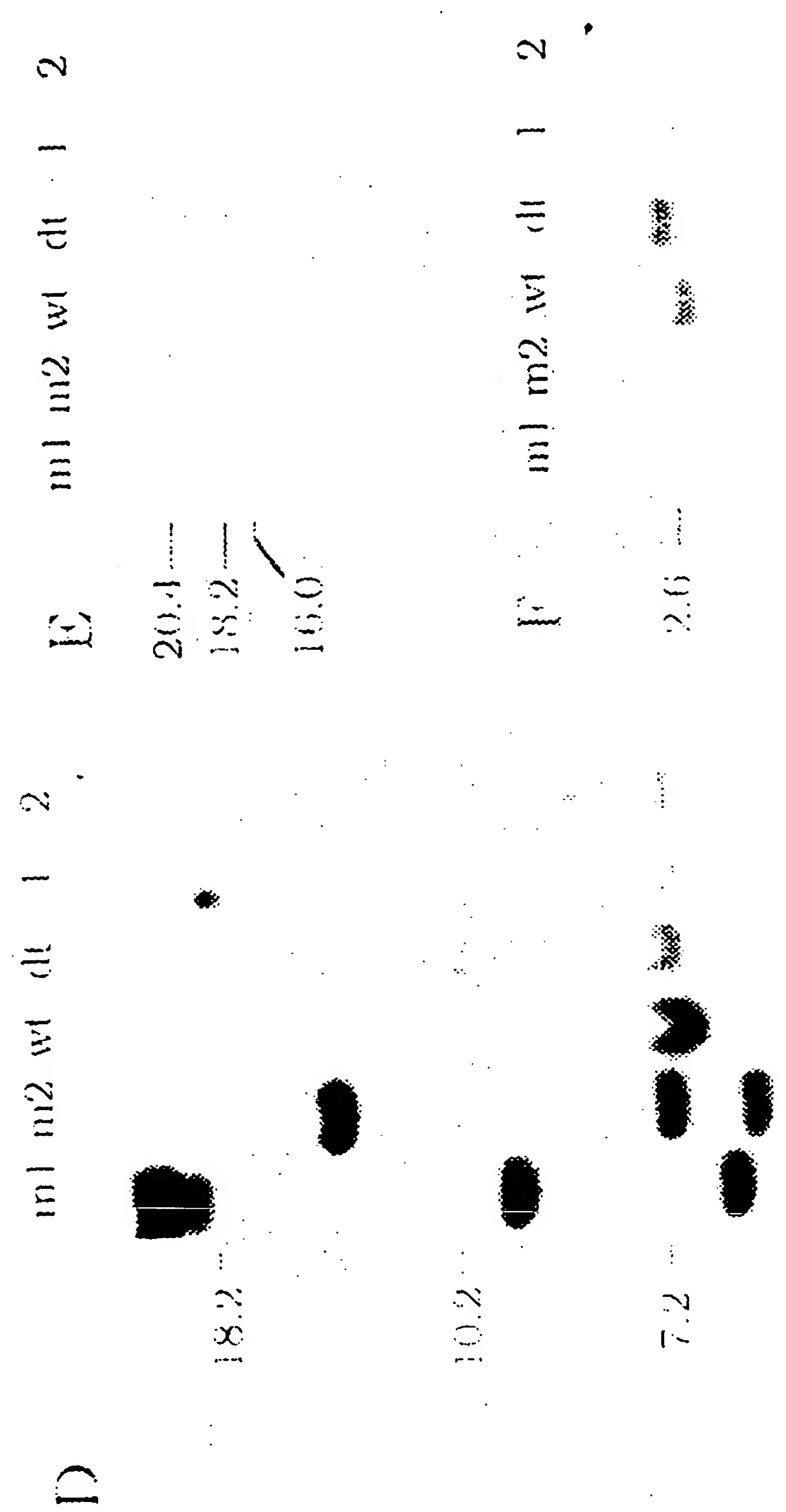
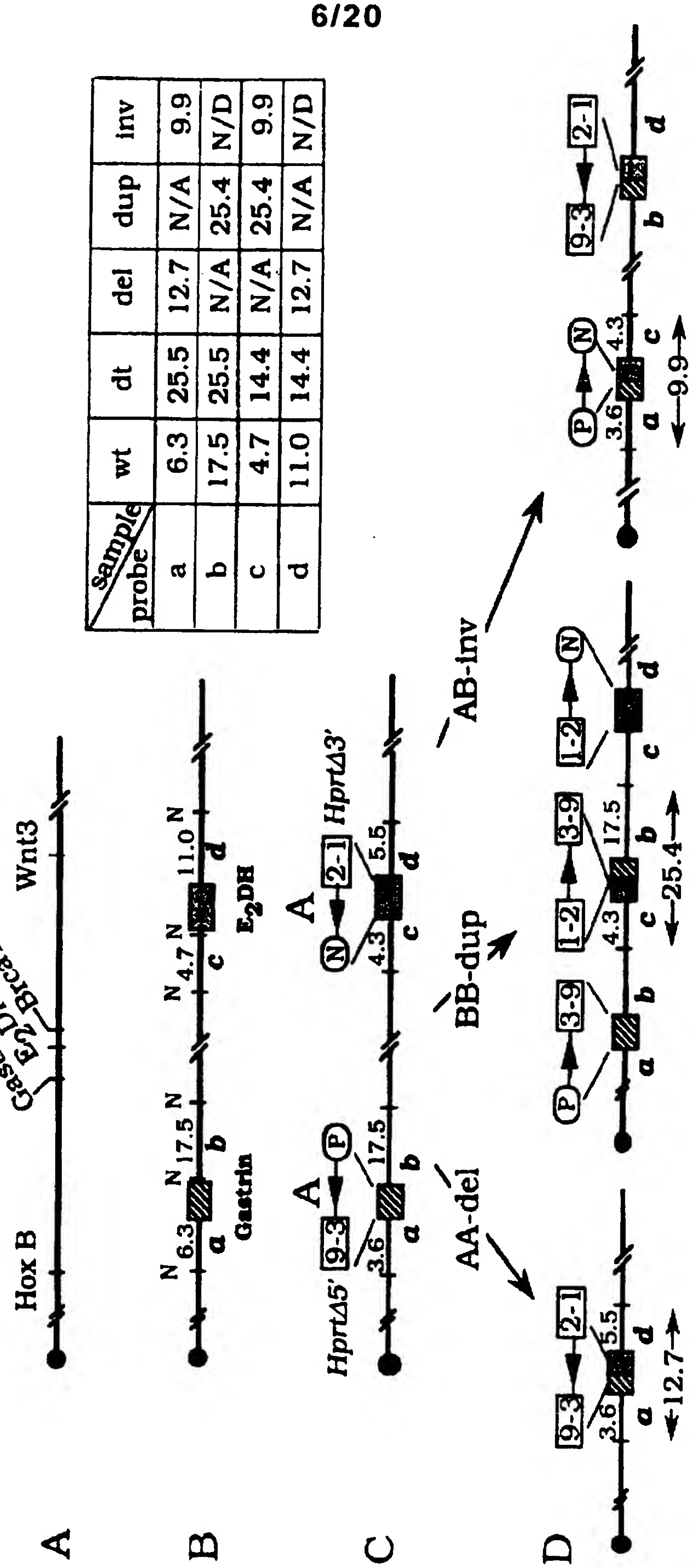

D
m1 m2 wt dt 1 2
18.2
10.2
7.2
E
m1 m2 wt dt 1 2
20.4
18.2
16.0
F
m1 m2 wt dt 1 2
2.6

Figure 2



sample / probe	wt	dt	del	dup	inv
a	6.3	25.5	12.7	N/A	9.9
b	17.5	25.5	N/A	25.4	N/D
c	4.7	14.4	N/A	25.4	9.9
d	11.0	14.4	12.7	N/A	N/D

Figur 3

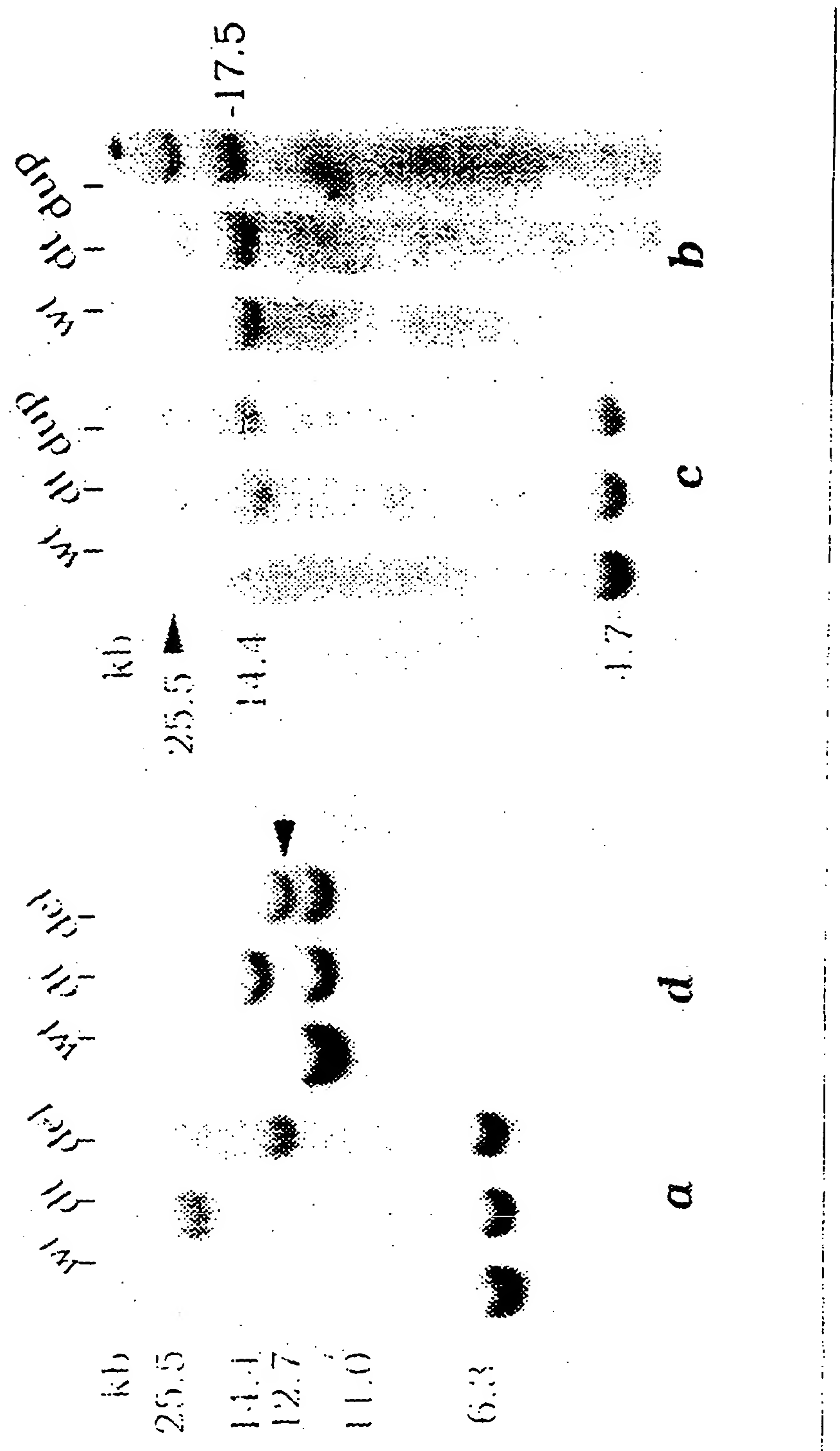


Figure 3E

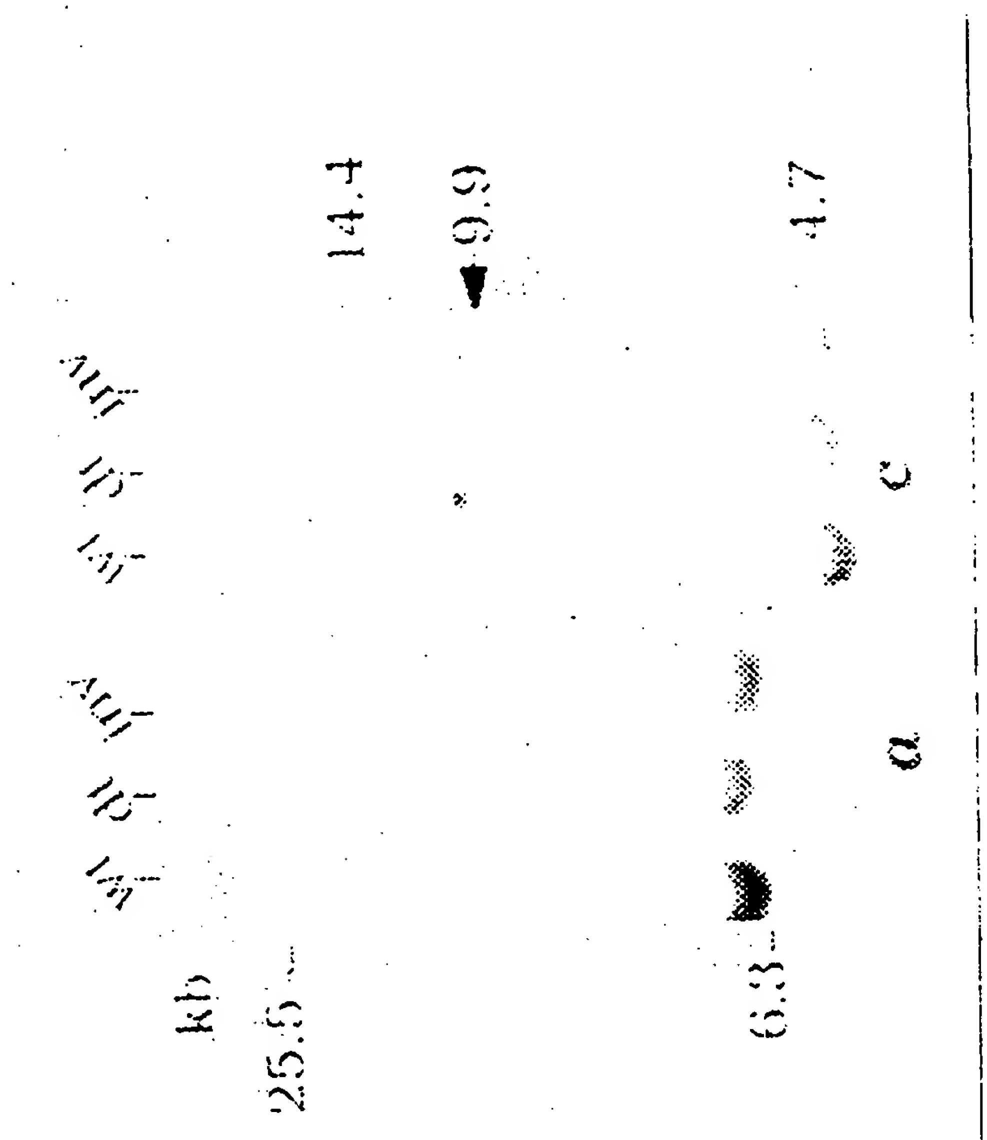


Figure 3E (Continued)

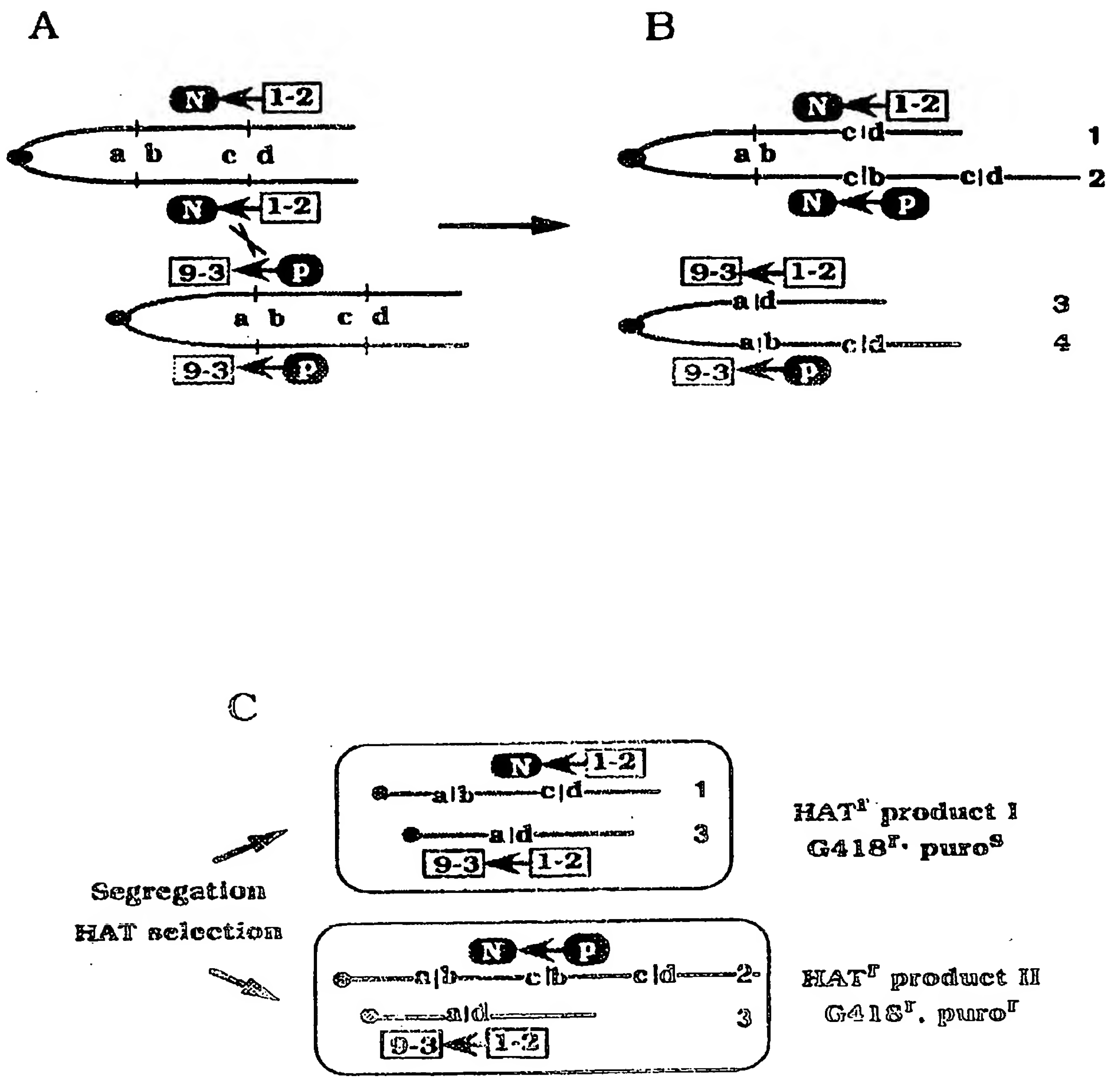

A
N
1-2
a b c d
N
1-2
9-3
P
a b c d
9-3
P
B
N
1-2
c|d
1
a b
c|b
c|d
2
N
P
9-3
1-2
a|d
3
a|b
c|d
4
9-3
P
C
N
1-2
a|b
c|d
1
a|d
3
9-3
1-2
Segregation
HAT selection
N
P
a|b
c|b
c|d
2
a|d
3
9-3
1-2
HAT^s product I
G418^r, puro^s
HAT^r product II
G418^r, puro^r

Figure 4

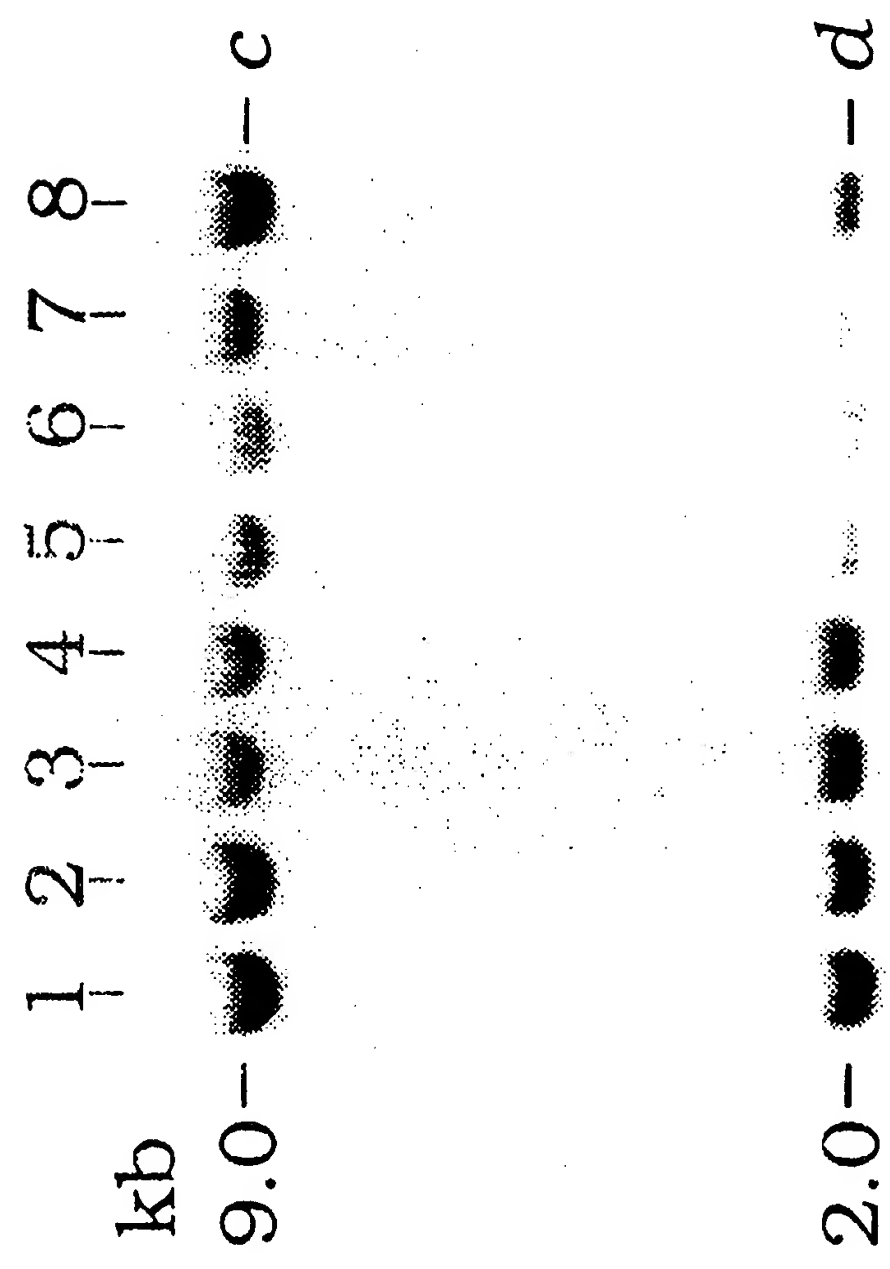


Figure 5

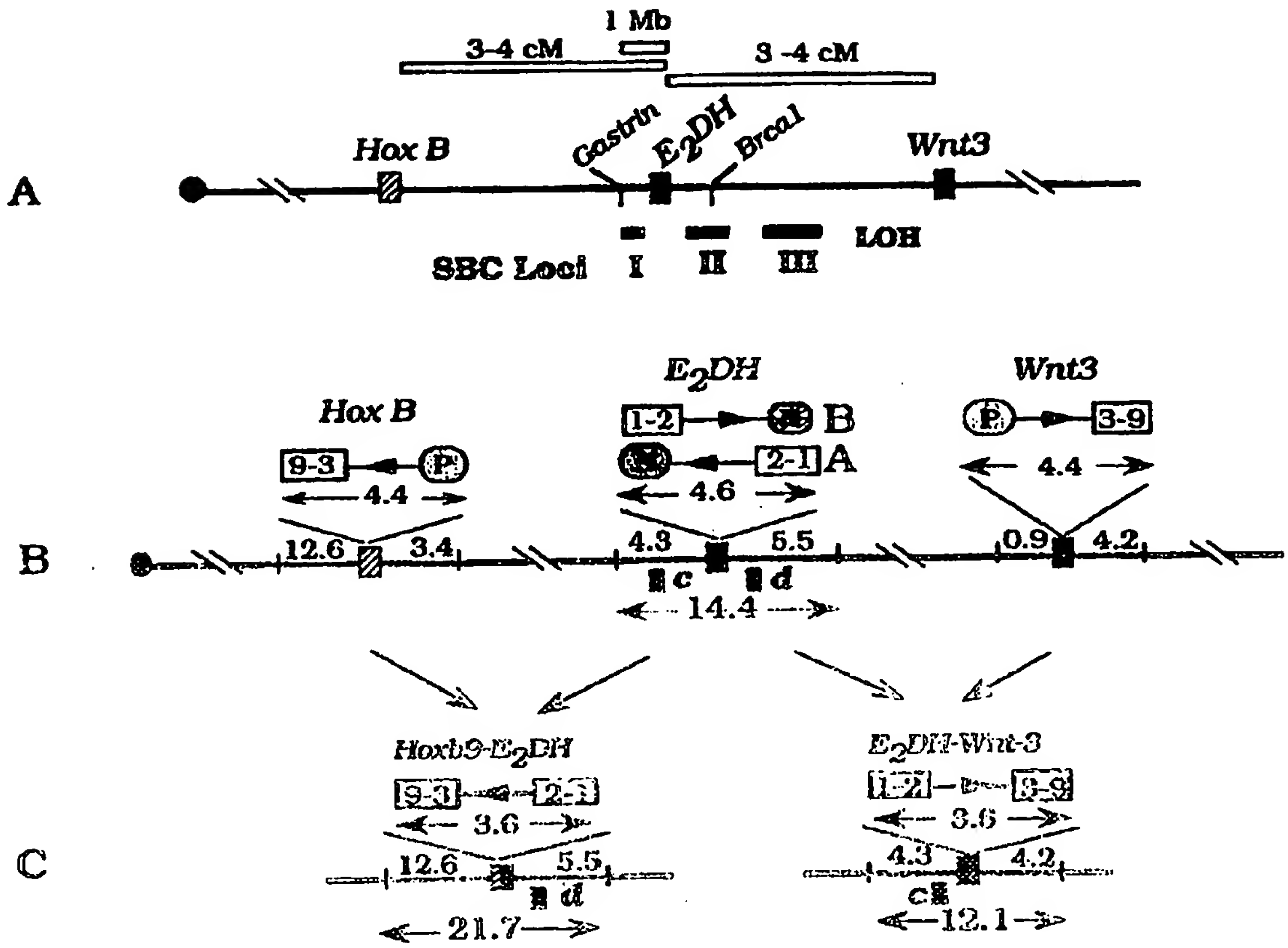


Figure 6

SUBSTITUTE SHEET (RULE 26)

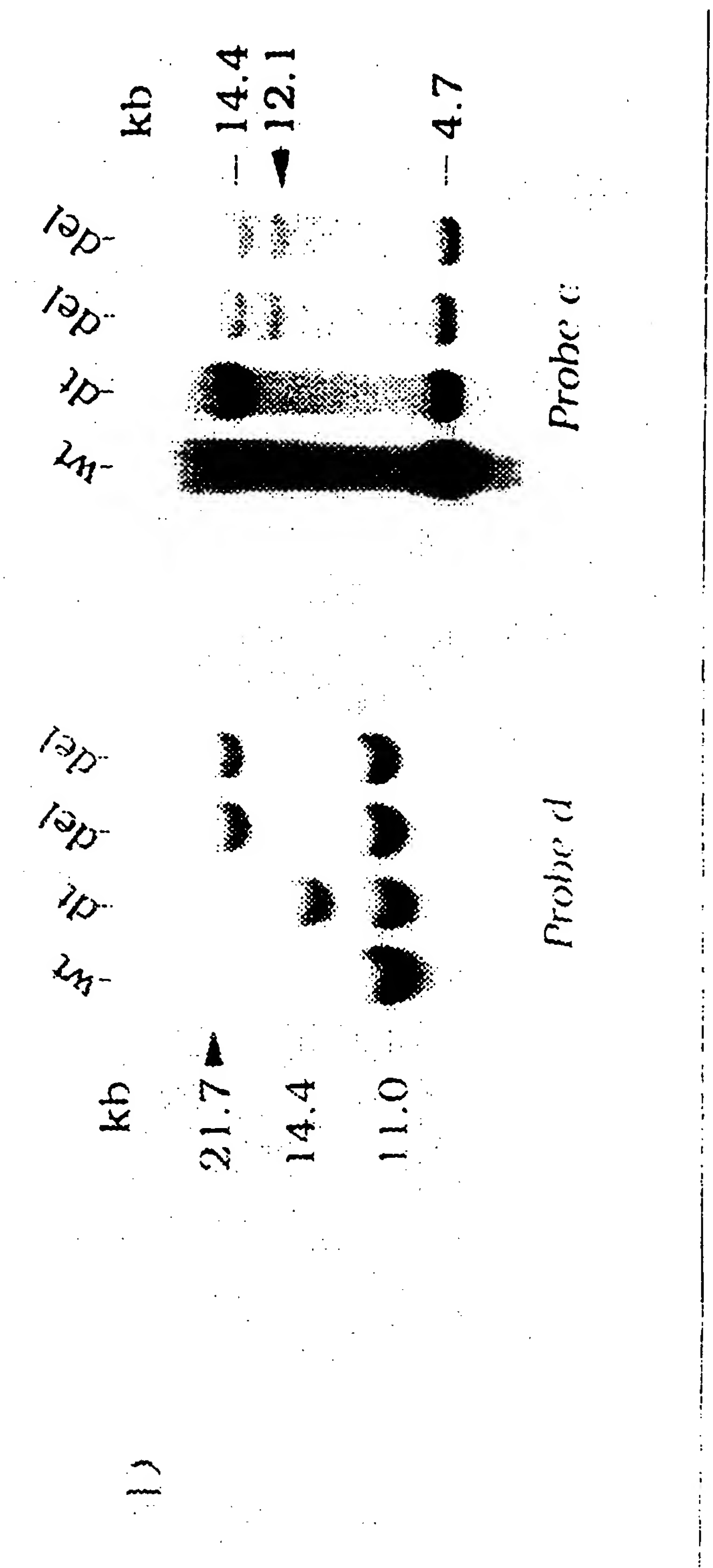
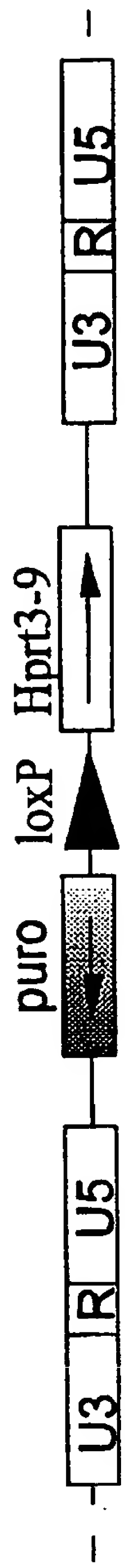
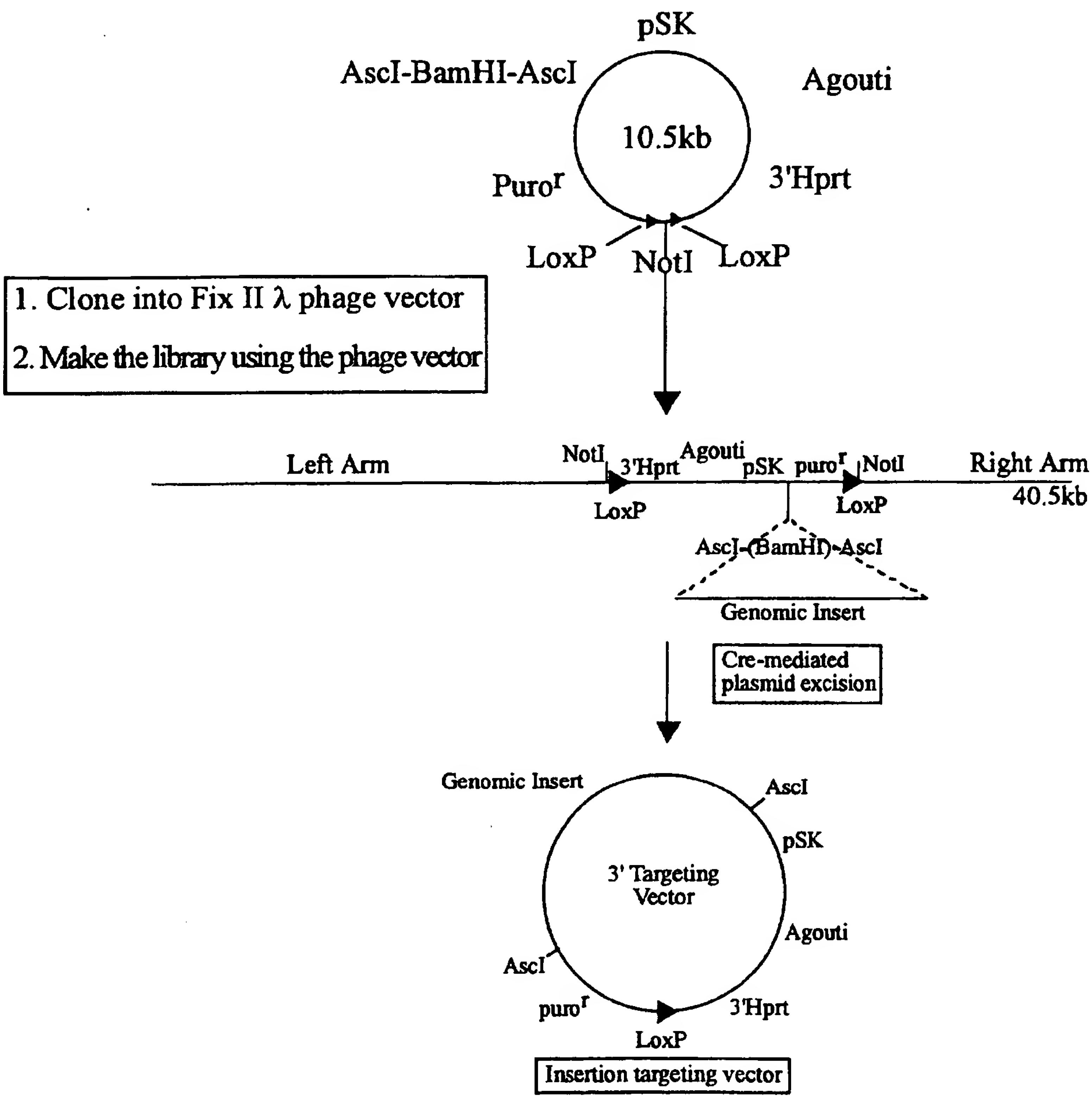


Figure 6

SUBSTITUTE SHEET (RULE 26)



Figur 7


pSK
AscI-BamHI-AscI
Agouti
10.5kb
Puro^r
3'Hprt
LoxP
NotI
LoxP
1. Clone into Fix II λ phage vector
2. Make the library using the phage vector
Left Arm
NotI
3'Hprt
Agouti
pSK
puro^r
NotI
Right Arm
40.5kb
LoxP
LoxP
AscI-(BamHI)-AscI
Genomic Insert
Cre-mediated plasmid excision
Genomic Insert
AscI
pSK
3' Targeting Vector
Agouti
AscI
puro^r
3'Hprt
LoxP
Insertion targeting vector

Figur 8

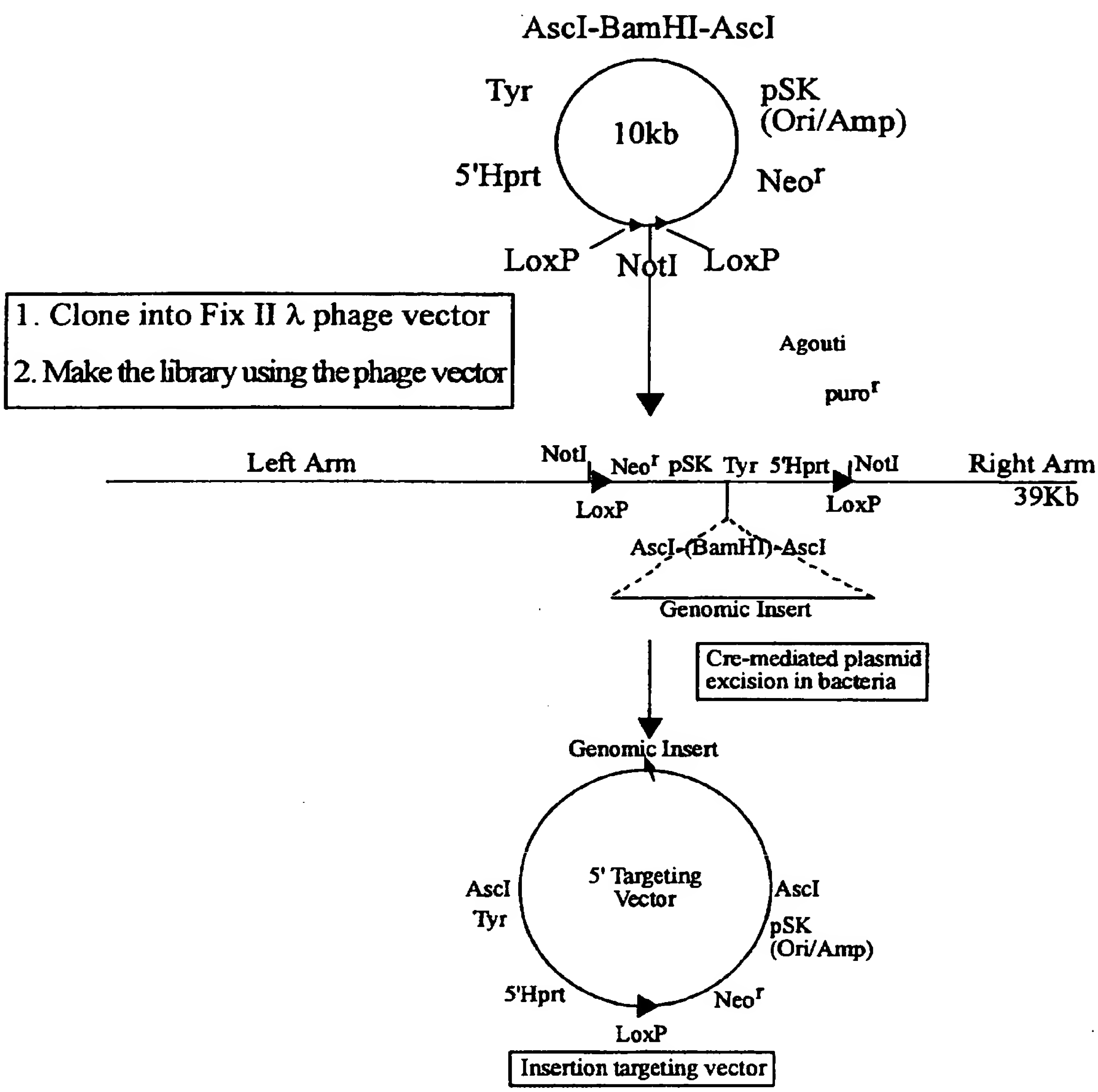

AscI-BamHI-AscI
Tyr
10kb
pSK
(Ori/Amp)
5'Hprt
Neor
LoxP
NotI
LoxP
1. Clone into Fix II λ phage vector
2. Make the library using the phage vector
Agouti
puror
Left Arm
NotI
Neor pSK Tyr 5'Hprt
NotI
Right Arm
LoxP
LoxP
39Kb
AscI-(BamHI)-AscI
Genomic Insert
Cre-mediated plasmid excision in bacteria
Genomic Insert
5' Targeting Vector
AscI
Tyr
AscI
pSK
(Ori/Amp)
5'Hprt
Neor
LoxP
Insertion targeting vector

Figure 9

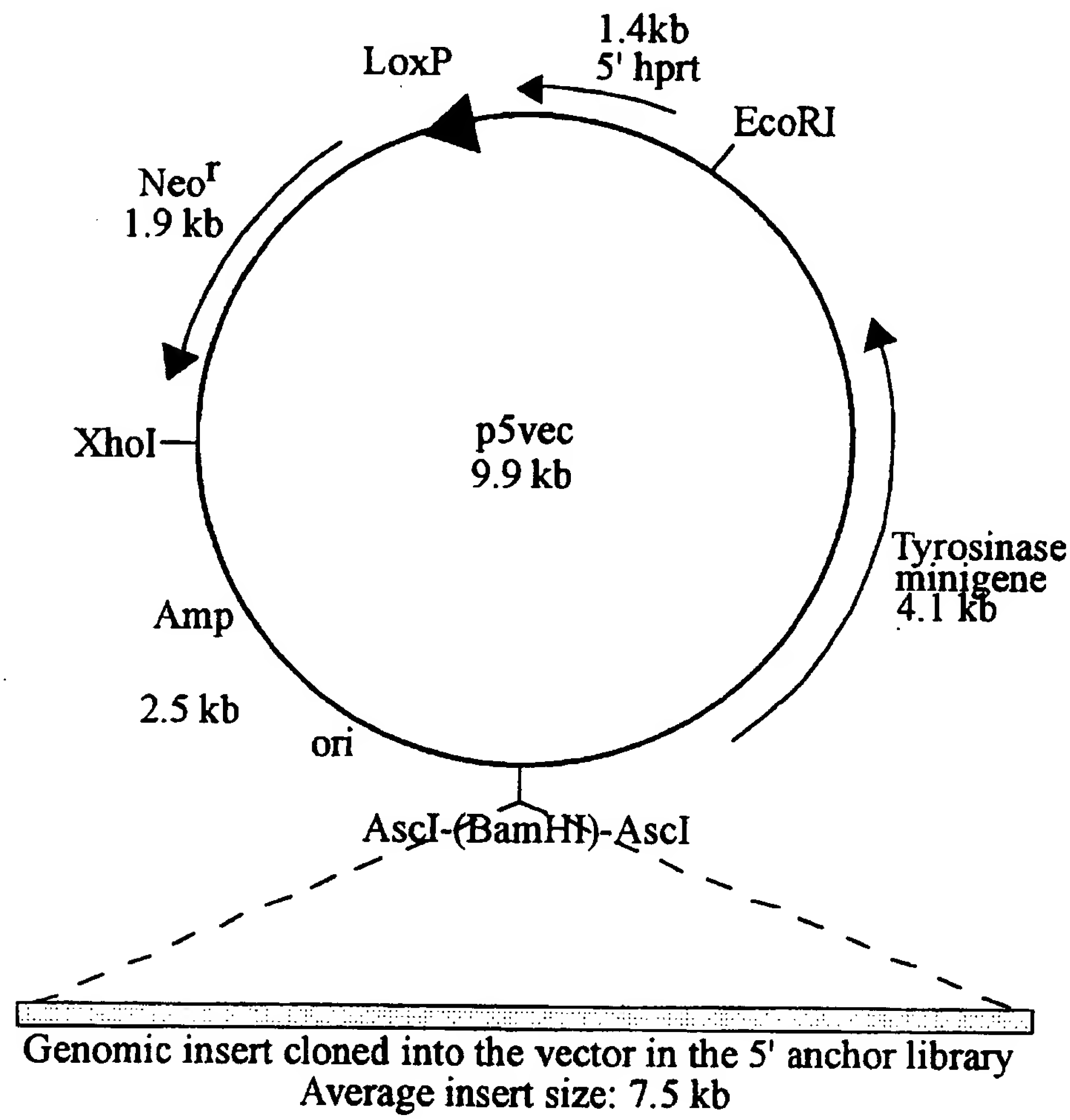

1.4kb
5' hprt
LoxP
EcoRI
Neo^r
1.9 kb
XhoI
p5vec
9.9 kb
Tyrosinase
minigene
4.1 kb
Amp
2.5 kb
ori
AscI-(BamHI)-AscI
Genomic insert cloned into the vector in the 5' anchor library
Average insert size: 7.5 kb

Figure 10

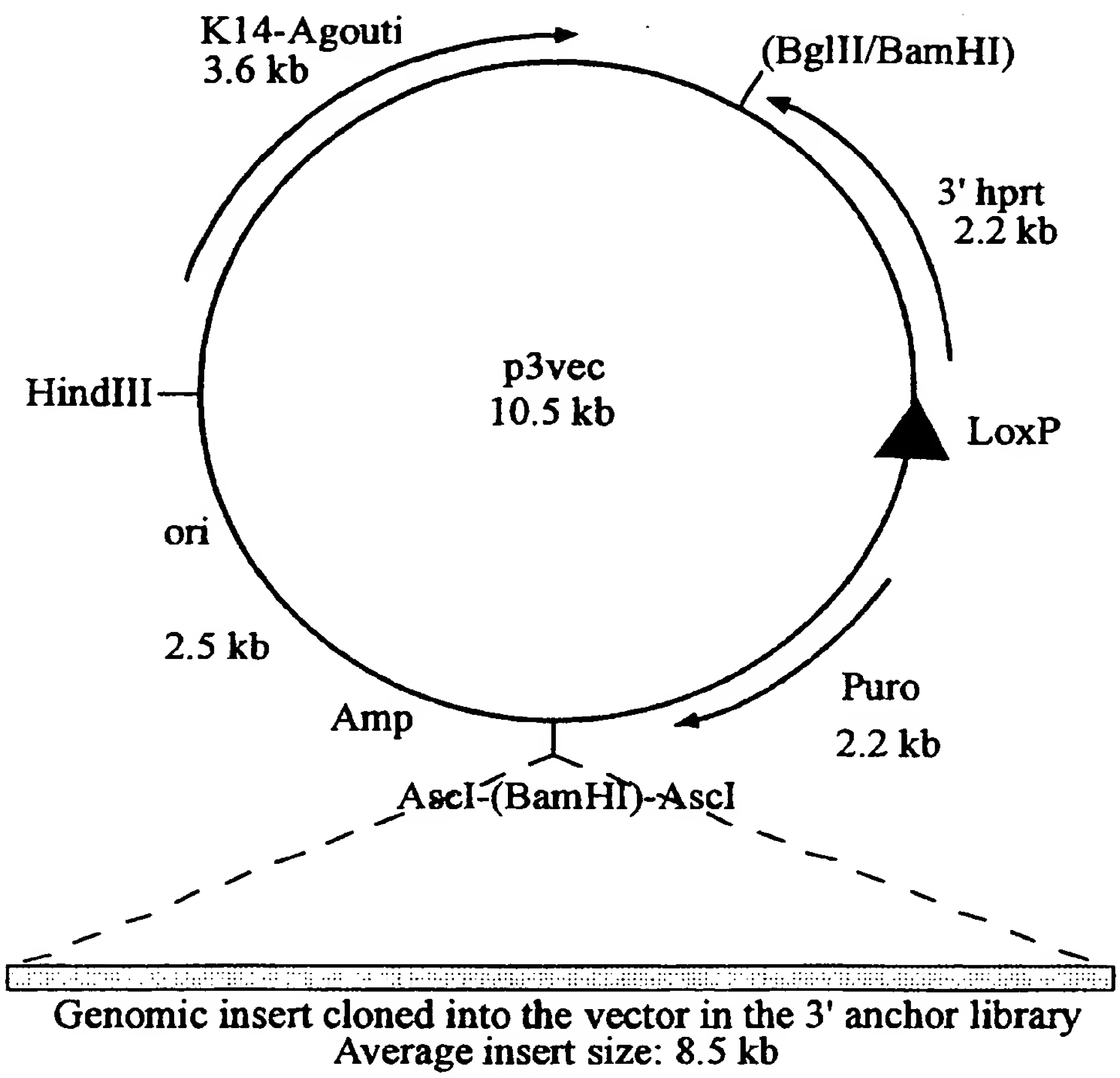

K14-Agouti
3.6 kb
(BglII/BamHI)
3' hprt
2.2 kb
p3vec
10.5 kb
HindIII
LoxP
ori
2.5 kb
Amp
Puro
2.2 kb
AscI-(BamHI)-AscI
Genomic insert cloned into the vector in the 3' anchor library
Average insert size: 8.5 kb

Figure 11

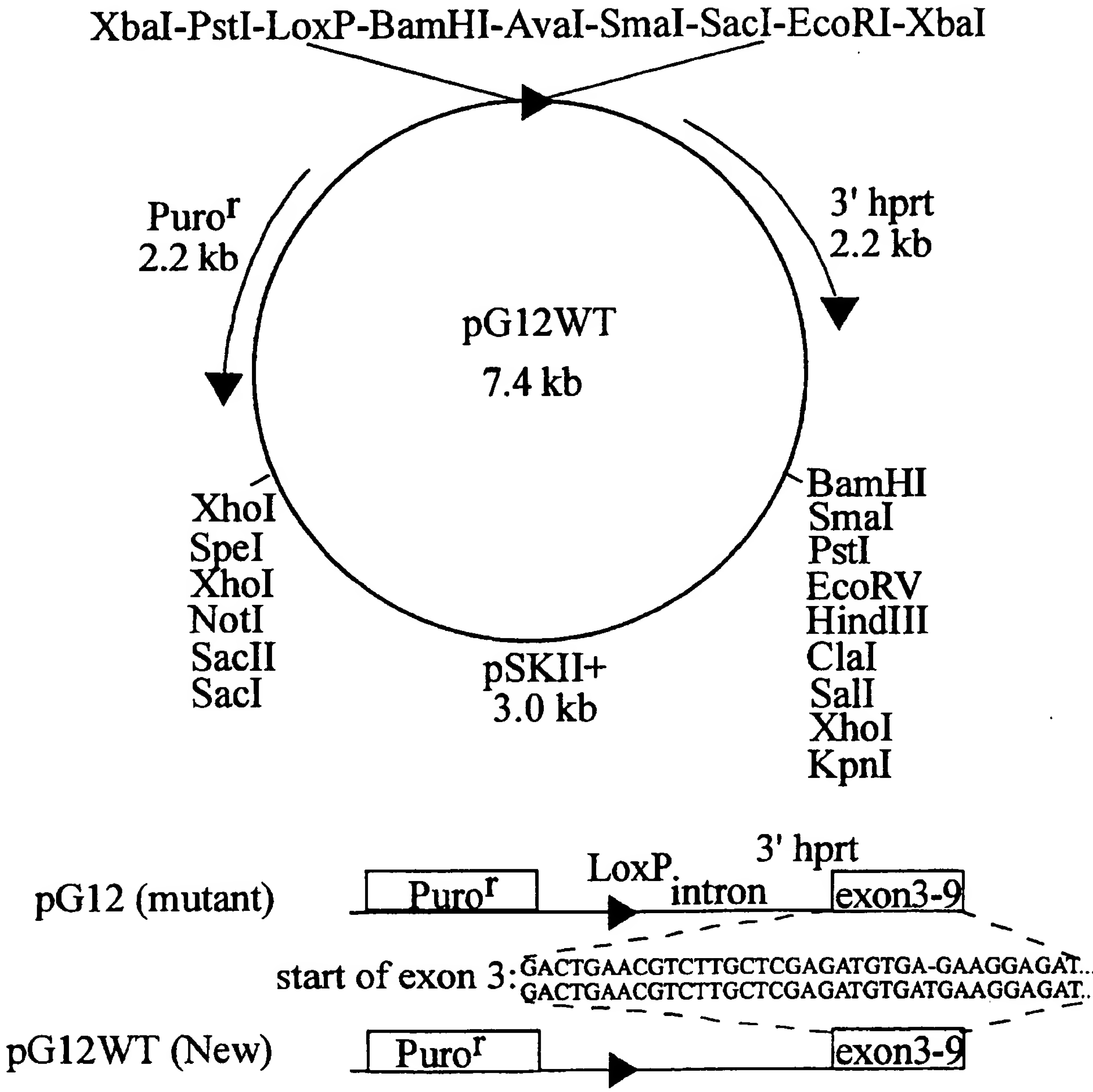
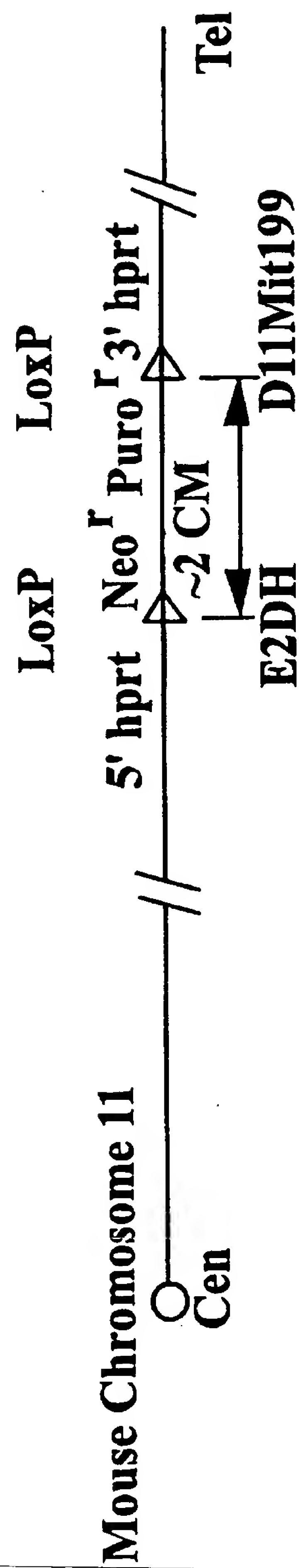

XbaI-PstI-LoxP-BamHI-AvaI-SmaI-SacI-EcoRI-XbaI
Puro^r 2.2 kb
3' hprt 2.2 kb
pG12WT
7.4 kb
XhoI
SpeI
XhoI
NotI
SacII
SacI
pSKII+ 3.0 kb
BamHI
SmaI
PstI
EcoRV
HindIII
ClaI
SalI
XhoI
KpnI
pG12 (mutant)
Puro^r
LoxP
intron
3' hprt
exon3-9
start of exon 3:
GACTGAACGTCTTGCTCGAGATGTGA-GAAGGAGAT...
GACTGAACGTCTTGCTCGAGATGTGATGAAGGAGAT...
pG12WT (New)
Puro^r
exon3-9

Figure 12


Tel
LoxP
3' hprt
Puro r
D11Mit199
~2 CM
LoxP
Neo r
5' hprt
E2DH
Mouse Chromosome 11
Cen

Figur 13

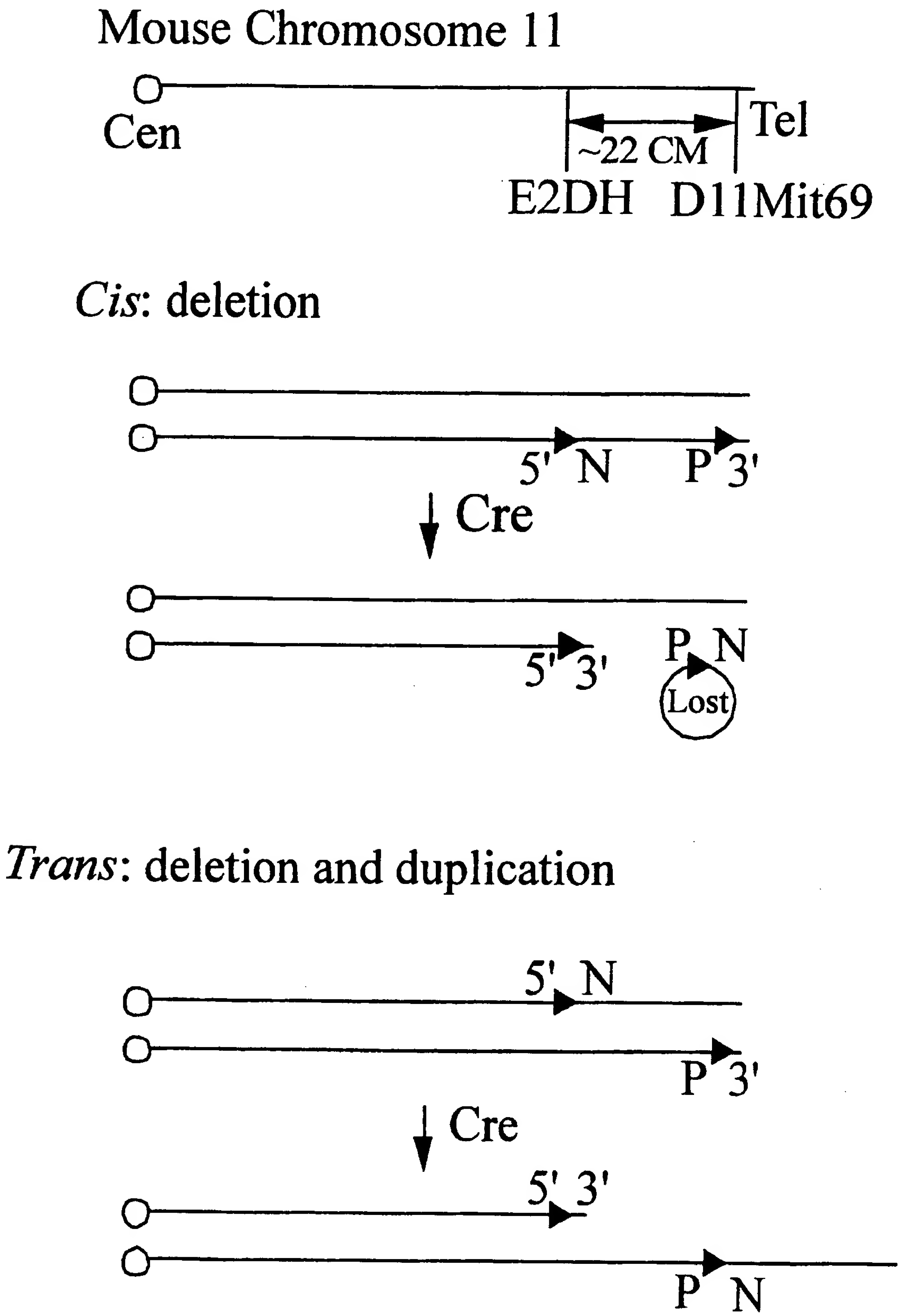

Mouse Chromosome 11
Cen
Tel
~22 CM
E2DH
D11Mit69
Cis: deletion
5'
N
P
3'
Cre
5'
3'
P
N
Lost
Trans: deletion and duplication
5'
N
P
3'
Cre
5'
3'
P
N

Figure 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/11143

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/00,15/11
US CL : 435/172.3, 320.1: 536/23.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 320.1: 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline, BIOSOS
search terms: cre, site specific recombination, retroviral vectors, tyrosinase and gene, K14 agouti

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	Ramirez-Solis et al. Chromosome engineering in mice. Nature. 14 December 1995, Vol. 378, pages 720-724, especially page 720.	1-9, 20 ---------- 10-18
Y	Gordon et al. Gene therapy using retroviral vectors. Current Opinion in Biotechnology. 1994, Vol. 5, pages 611-616, especially page 613.	12-16
Y	Qin et al. Cre recombinase-mediated site-specific recombination between plant chromosomes. Proc. Natl. Acad. Sci. USA. March 1994, Vol. 91, pages 1706-1710, especially pages 1707-1709.	1-20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 SEPTEMBER 1997	29 OCT 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	JOHN S. BRUSCA
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/11143

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Larue et al. Pigmented Cell Lines of Mouse Albino Melanocytes Containing a Tyrosinase cDNA with an Inducible Promoter. Somatic Cell and Molecular Genetics. 1990, Vol. 16, Number 4, pages 361-368, especially pages 363-366.	
Y	Lakso et al. Targeted oncogene activation by site-specific recombination in transgenic mice. Proc. Natl. Acad. Sci. USA. July 1992, Vol. 89, pages 6232-6236, especially pages 6233-6235.	16-18
Y	Kucera et al. Overexpression of an Agouti cDNA in the Skin of Transgenic Mice Recapitulates Dominant Coat Color Phenotypes of Spontaneous Mutants. Dev. Biol. January 1996, Vol. 173, pages 162-173, especially pages 164-170.	16-18
Y	Gossen et al. Transcriptional Activation by Tetracyclines in Mammalian Cells. Science. 23 June 1995, Vol. 268, pages 1766-1769, especially pages 1766-1769.	10
Y	Capecchi. Altering the Genome by Homologous Recombination. Science. 16 June 1989, Vol. 244, pages 1288-1292, especially pages 1289-1290.	2, 4, 14-20
Y	Smith et al. A site-directed chromosomal translocation induced in embryonic stem cells by Cre-loxP recombination. Nature Genetics. April 1995, Vol. 9, pages 376-385, especially pages 376-379.	1-20